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Cirugía de ojo seco y cataratas

Dry eye and cataract surgery

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PRESENTADA POR

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ABSTRACT

Cataract surgery, one of the most frequently performed surgical procedures in the world, is safe and efficacious, however, it is a risk factor for dry eye disease which can lead to dissatisfaction with the outcome and can adversely impact quality of life. This thesis explores possible mechanisms whereby ophthalmic surgery could impact on the ocular surface and, in turn, lead to dry eye signs and symptoms postoperatively. Contributing factors may include light from the operating microscope, disruption of ocular tissues and antiseptic agents used such as povidone iodine.

This research attempted to understand the how cataract surgery affects the ocular surface by using an in vitro model. Porcine conjunctival fibroblasts were assessed after exposure to some of the stressors associated with surgery to observe the possible damage with three different approaches.

Firstly, an in vitro model was developed to assess the effect of the light exposure from an operating microscope. Preoperative and postoperative dry eye approach using hyperosmolar stress as a dry eye simulator, were observed to assess whether light exposure had an effect. Light exposure of 10 minutes slowed down the wound healing, increased inflammation, apoptosis rate and decreased cell viability. A greater increase in inflammation, apoptosis rate and delayed healing response was observed when the cells were under hyperosmolar stress.

Secondly, porcine conjunctival fibroblasts were exposed to ultraviolet radiation with different filters reflecting the fact that ultraviolet radiation either from the operating microscope and/or from environmental sources can potentially cause or worsen dry eye. Therefore, this study investigated the effect of radiation using different UV-A filters on the conjunctival fibroblasts. The cell viability was more severely affected by the ultraviolet radiation when the cells were under hyperosmolar stress, suggesting that the dry eye condition can be worsened by UV radiation.

Furthermore, a frequently preferred antiseptic agent, povidone iodine was adversely affect the cell viability. Concentration from 5% to 1% concentration of povidone iodine was also found to delay the wound healing with 3-minute exposure. The effect of reduced exposure time e.g. 30 seconds, 1 minute

and 2 minute on viability was also investigated; higher than 0.5% concentration of povidone iodine was found to be still toxic to the cells even with 30 seconds exposure.

To conclude, this in vitro model with different stressors allowed the investigation of a variety of different aspects of cataract surgery in order to better understand the causes of post-operative dry eye. Light exposure, and/or exposure to chemical agents like povidone iodine can potentially cause/worsen dry eye symptoms in these patients. In conclusion, both visible light from operating microscopes, agents such as povidone iodine and UV-A exposure are potential factors that can affect wound healing, decrease the cell viability and cause worsening of dry eye symptoms after cataract surgery.

SPANISH ABSTRACT

La cirugía de cataratas , uno de los procedimientos quirúrgicos realizados con más frecuencia en el mundo, es segura y eficaz. Sin embargo, este tipo de cirugía es un factor de riesgo para la enfermedad del ojo seco que puede conllevar a una insatisfacción con el resultado postquirúrgico y puede afectar negativamente a la calidad de vida del paciente.

En la presente tesis se ha utilizado un modelo in vitro para explorar los posibles factores por los cuales la cirugía oftálmica podría impactar en la superficie ocular y, a su vez, conducir a signos y síntomas postquirúrgicos que son característicos del ojo seco. Estos incluyeron la exposición a la luz del microscopio quirúrgico y el uso de agentes antisépticos tales como la povidona yodada. El modelo in vitro fue creado por fibroblastos de la conjuntiva porcina e incluyó dos escenarios de ojo seco, uno preoperatorio y otro postoperatorio. Ambos fueron simulados por un medio hiperosmolar, que se usó o bien antes o bien después de aplicar los factores estresantes asociados a la cirugía. Los resultados mostraron que el escenario de ojo seco preoperatorio, comparado con el de ojo postoperatorio, sufrió una ralentización en la cicatrización de la incisión, una disminución en la viabilidad celular y un aumento en la inflamación celular y tasa apoptótica tras diez minutos de exposición a la luz con un microscopio quirúrgico. Los rayos UV de la luz del microscopio quirúrgico fueron un factor importante que contribuyó a la disminución en la viabilidad celular. Así mismo, el uso de 5% a 1% de povidona yodada retrasó la cicatrización de la incisión tras tres minutos de su aplicación. Curiosamente, una exposición de 30s a 0.5% de povidona yodada permaneció siendo tóxico para células.

En conclusión, tanto la luz visible de los microscopios quirúrgicos, como la povidona yodada y la exposición al UV son factores potenciales que pueden afectar a la cicatrización de las heridas, disminuir la viabilidad celular y causar un empeoramiento de los síntomas del ojo seco después de la cirugía de cataratas.

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ASSOCIATED PUBLICATIONS

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LIST OF ABBREVIATIONS

CCL2	Chemokine (C-C motif) ligand 2
CMC	Carboxymethylcellulose
DEWS	Dry Eye Workshop
DMEM	Dulbecco's Modified Eagle's medium
DMSO	Dimethyl Sulfoxide
EDTA	Ethylenediaminetetraacetic acid
EthD-1	Ethidium Homodimer
FBS	Foetal Bovine Serum
HLA-DR	Human Leukocyte Antigen – DR isotype
Hy-A	Hyaluronic Acid
IL	Interleukin
IOL	Intraocular Lens
LG	L-glutamine
MAPK	Mitogen-Activated Protein Kinases
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide
NaCl	Sodium Chloride
NSAID	Nonsteroidal Anti-Inflammatory Drugs
OSDI	Ocular Surface Disease Index
PBS	Phosphate Buffered Saline
PVI	Propidium Iodide (PVI)
PTB	Physikalisch-Technische Bundesanstalt
PVI	Povidone Iodine
TCP	Tissue Culture Polystyrene
TFLL	Tear Film Lipid Layer (TFLL)
TFOS	Tear Film and Ocular Surface
TNF-α	Tumour Necrosis Factor Alpha
UV	Ultraviolet Radiation
VEGF	Vascular Endothelial Growth Factor

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OUTLINE OF THE THESIS

This thesis consists of 7 chapters. The first chapter defines the background information about the ocular surface, cataract surgery and dry eye disease pathology followed with a review of the literature about studies investigating dry eye after cataract surgery and its aetiology. A review of in vitro models of the dry eye used to explore the aetiology of dry eye after cataract surgery is included. Lastly, the gaps in the literature and how this thesis attempts to fill these gaps is summarized.

The second chapter outlines the common methodology used in this thesis, along with a list of materials and instruments and a detailed assessment of the procedures used throughout the thesis.

The third chapter describes the development of an in vitro model of dry eye. This model was used to assess the phototoxicity from an operating microscope which could potentially cause or worsen dry eye symptoms postoperatively. A variety of approaches was used including assessment of cell viability, wound healing, inflammation level, apoptosis rate.

The fourth chapter studied the effect of UV irradiation with different wavelengths with an in vitro model. This chapter explored whether cell viability and cell size are affected more by the UV irradiation in the presence of hyperosmolar stress in vitro.

The fifth chapter investigated the effect of povidone iodine, a commonly used antisepsis agent in ophthalmic surgery, on conjunctival fibroblasts. A variety of different concentration of povidone iodine, along with the altered exposure times were tested to observe cell viability differences on conjunctival fibroblast. The wound healing response was observed on monolayer cultures to interpret the previous statements about the povidone iodine. Three-dimensional culture was also constructed to better mimic the in vivo conditions to observe the penetration of povidone iodine.

The sixth chapter summarizes the findings of the overall thesis and highlights the impact of this research's results. Finally, the seventh chapter states the limitations of the overall thesis and informs about the future directions.

CHAPTER 1: LITERATURE REVIEW

1 LITERATURE REVIEW

Overview

Dry eye disease is one of the most common ocular surface pathologies, affecting more than 5% of the worldwide population [1]. The latest DEWS TFOS Report (2017) defines dry eye as:

“a multifactorial disease of the ocular surface characterized by a loss of homeostasis of the tear film, and accompanied by ocular symptoms, in which tear film instability and hyperosmolarity, ocular surface inflammation and damage, and neurosensory abnormalities play etiological roles.”

Researchers around the world are constantly advancing the understanding of this disease and, recently, cataract surgery has been indicated as one of the key risk factors for the dry eye [2]. In particular, it has been shown that patients undergoing ocular surgery, are characterized by higher chances (up to 70 %) of developing dry eye as well as experience worsening of their pre-existing dry eye conditions [3]. Nevertheless, the precise mechanisms for the development of dry eye after cataract surgery are still unknown. Possible causative factors of the postoperative dry eye may include the

surgical incision, the light exposure coming from the operating microscope, the cytotoxicity of the antiseptic agents, and topical medications used before, during and/or after the procedure.

All these factors can potentially affect the ocular surface homeostasis after the surgery, and this chapter reviews the relevant literature to better elucidate the correlation between the cataract surgery and dry eye. Anatomy of the ocular surface; is firstly described providing background information on the current understanding of dry eye disease pathophysiology, followed with the aetiology of dry eye after surgery. Lastly, in vitro dry eye models focused on hyperosmolarity and inflammation as the main mechanisms of dry eye are discussed to highlight the key factors to take into account when setting up in vitro models of dry eye.

1.1 Anatomy of the Ocular Surface

The ocular surface comprises the entire conjunctiva from the lid margin to posterior part of the eyelids, cornea, limbus and the tear film. Collectively, these structures are called the anatomical ocular surface, a term firstly coined by Thoft in 1978 [4]. The functional unit of the ocular surface comprises cornea, conjunctiva, the eyelids, lacrimal and meibomian glands, tear film, muscles required for eye movement and blink reflexes [5,6]. All the structures of the anatomical unit of the ocular surface are designed to control and preserve the healthy ocular environment to ensure clear vision [5,7].

1.1.1 Tear Film

The tear film is a complex and dynamic structure mainly acting as a protecting barrier while being responsible for the hydration homeostasis of the ocular surface. Historically, the tear film was considered to be a three-layered structure: mucin layer (2.5-5 µm) [8], aqueous layer (2.17-4µm) [8,9] and lipid layer (20–150 nm) [10]. However, evidence has been indicating that the mucin layer is actually distributed in the aqueous phase with a gradient concentration, forming a mucoaqeous layer with a thickness of 2-6 µm that makes the most of the bulk of the tear film [8,9,11–13]. Mucins play a role as a safeguard for epithelial cell layers against infection or damage [14]. The aqueous phase of the tear film is composed of proteins, salts, growth factors, hormones, cytokines, anti-microbial agents,

vitamins and gel-forming mucins [15,16]. Lastly, the tear film lipid layer (TFLL) is reported to have a mean thickness of around 42nm [10] and consists of lipids which are mainly secreted by the meibomian glands. The lipid layer is responsible for controlling the evaporation rate, especially in the open eye, ensuring the stability of the tear film and a smooth ocular surface [17]. In addition, the lipid layer has the combination of polar and nonpolar lipids containing different lipid types, with over 1500 different types of proteins that help to reduce the aqueous surface tension to further stabilise the tear film stabilityError! Reference source not found. [18,19].

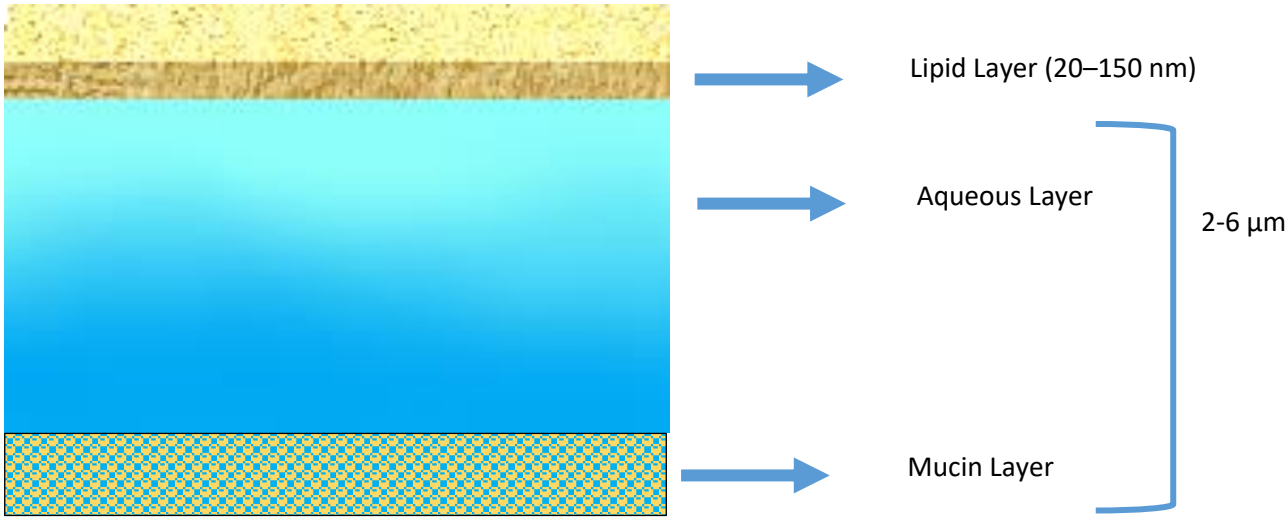

Figure 1-1.Tear film structure. The lipid layer is the most outermost layer of the tear film. The aqueous layer is shown schematically with some of the mucins. The innermost layer is the mucin layer.

1.1.2 Cornea

The cornea is very complex and a unique tissue of the human body consisting of five different layers anisotropically arranged to ensure corneal transparency, guarantee refraction capacity and to protect the internal structures of the eye [20]. It is an immune-privileged site due to the absence of lymphoid cells and blood vessels within it, which is extremely favourable during the ophthalmic surgical incisions [21]. The restricted immune response allows the surgical incisions to heal without scarring and limiting contracture which can lead to a change of corneal shape [21,22]

The epithelium is the outermost layer of the cornea and contains five to seven layers of epithelial cells [23]. The epithelial cells have the capacity to be renewed every two weeks from limbal stem cells which can differentiate [24,25]. In the case of wound healing or any epithelial trauma, such as incisions due to ocular surgical procedures, the healing process accelerates and recovers very quickly within hours [26].

Bowman's Layer is the acellular layer located between the stroma and the corneal epithelium. This thin layer mostly consists of collagen, which is randomly orientated and also thinner than any other collagen found in the cornea [27]. The most prominent collagen types present in this layer are type I, III and V [28].

The stroma is located between Bowman's Layer and Descemet's Layer. The stroma mainly consists of keratocytes which have a quiescent, dendrite shape. Corneal stroma consists of highly organised collagen fibrils which are important in maintaining corneal transparency [29,30]. The main matrix components are collagen type I, V and glycosaminoglycans such as keratan sulfate, dermatan sulfate, several proteoglycans and also other proteins like fibronectin and laminin [23,30]. The stroma plays a significant role in maintaining corneal transparency and mechanical strength due to the spatial arrangement of the collagen fibrils within it. In particular, parallel arrays are found in those fibrils which gives the corneal stroma its unique water-holding feature [21].

Descemet's membrane is the tissue separating the stroma and endothelial layer. It is elastic and increases its thickness with age [31]. It is composed of widely spaced collagen type fibrils [32].

The innermost layer of the cornea is the endothelial layer. It has a fundamental role in maintaining the ocular visual function as it regulates the ion transportation between the stroma and anterior chamber, maintaining corneal hydration [33]. This layer consists of 5 to 7 sided cuboidal endothelial cells which are highly specialised [34]. The endothelium is essential for the ion transportation system which maintains the hydration of the stroma. One of the important features of those cells is that they have no capacity to proliferate [35].

1.1.3 Conjunctiva

The conjunctiva is a transparent mucous membrane surrounding the inner eyelids and the eyeball up to the corneoscleral limbus. It is divided into three regions; palpebral, forniceal and the bulbar conjunctiva [36]. The palpebral conjunctiva covers the inner part of the eyelid. The forniceal conjunctiva is located in the folding area between the globe and eyelids and lines from the bulbar conjunctiva to the palpebral conjunctiva [37]. The bulbar conjunctiva covers the anterior part of the sclera, until the junction between cornea and sclera, binding to Tenon's capsule and the extraocular muscle insertions [38].

The conjunctiva has a thickness of 240.1 ± 29.8 µm containing a stratified squamous epithelial cell layer 42.4 ± 7.4 µm thick and an underlying conjunctiva stroma 197.7 ± 32.5 µm thick [36,39]. The epithelial cell layer consists of cuboidal epithelial cells along with goblet cells while the stroma contains fibroblasts, lymphocytes, Langerhans and melanocytes [36]. Conjunctival epithelial cells protect the ocular surface from the external world [39]. There is also debate about the conjunctival epithelial stem cells which are believed to be in the conjunctival tissue itself however the discussion is still ongoing about the exact location of those cell types [37,40]. Goblet cells are responsible for producing mucin for the tear film and resisting the shear stress during blink or saccade [41,42].

The conjunctival stroma, on the other hand, is highly vascularised and consists of two different layers; a layer with lymphocytes and a thicker fibrous layer with blood vessels and nerves, which play a significant role in inflammation and fibrosis [39]. In fact, it is the first location to generate the ocular inflammation response due to the presence of highly interconnected inflammatory cells. In addition, the conjunctival stroma provides an additional protective function to the cornea by being optimised for providing a more intense response to inflammation than the cornea which is instead an immune privileged site [43,44]. Therefore, healthy conjunctiva is crucial for successful ocular surface repair and for maintaining the homeostasis of the ocular surface [45].

1.1.4 Meibomian Glands

Meibomian glands are holocrine glands, located in the superior and inferior tarsal plates [46]. They are responsible for producing meibomian lipids or meibum. The secretion is transferred to the lid margin and combined with the tears and spread over the ocular surface through each blink [47]. The meibomian orifices can be found in both the upper and lower eyelid margins and they play a vital role in tear film instability by preventing evaporation [48]. The meibum consists of nonpolar and polar lipids covering the aqueous layer of the tear film [18]. In summary, the lipids are responsible for providing a smooth surface at the air-lipid interface, helping decrease tear evaporation, improving the stability of tear film, and helping to seal the eyelids properly during sleep [48,49].

1.2 Dry Eye

Dry eye disease was previously believed to be caused by a tear film disorder [50]. There has been some controversy over the last few decades about dry eye disease and its definition. In fact, until 2007 [51], it was not even described as a disease. Recently, the DEWS II provided a precise definition of dry eye as: "a multifactorial disease of the ocular surface characterized by a loss of homeostasis of the tear film, and accompanied by ocular symptoms, in which tear film instability and hyperosmolarity, ocular surface inflammation and damage, and neurosensory abnormalities play etiological roles."[1]. Hyperosmolarity of the tear film and inflammation of the ocular surface are the main mechanisms of dry eye disease. However, despite the large number of studies published on dry eye, the disease is not yet fully understood.

Dry eye disease has been divided into two categories, aqueous deficient dry eye and evaporative dry eye. This classification is mostly based on the cause of dry eye. Aqueous deficient dry eye is related to disorders of the lacrimal glands or lacrimal tear secretion, whereas evaporative dry eye is caused by an increased loss of tear fluid from the ocular surface, predominantly due to meibomian gland dysfunction [52]. In addition to the two types of dry eye classification, many hybrid forms of the dry eye also exist [53].

Aqueous tear-deficient dry eye is divided into two subtypes, Sjögren's syndrome and non-Sjögren's syndrome dry eye. Sjögren's syndrome, an autoimmune disorder, affects the exocrine glands in the body [54]. Activation of immune cells and the production of antibodies against the body's own proteins occurs as part of the disease mechanism [54]. Sjögren's syndrome affects not only the lacrimal glands but also other glands like salivary glands and it is a systemic progressive autoimmune disease [52,54]. For these reasons, patients complain not only of dry eyes but also of dry mouth. Inflammation of the lacrimal glands and the cytokines in the tear film and conjunctiva leads to reduced aqueous tear production and dry eye [52,55,56]. Non-Sjögren's syndrome dry eye is not an autoimmune disease but is mostly related to age, which results in tear film deficiency, tear instability, reduced tear flow and reduced tear volume [57].

Evaporative dry eye is predominantly induced by meibomian gland dysfunction, which leads to the reduced or complete destruction of lipid production and results in excessive evaporation of the aqueous layer [52,57]. Evaporative dry eye can be initiated by intrinsic or extrinsic factors. Meibomian oil deficiency, lid aperture disorders, slow blink rate and drug effects are the intrinsic causes of evaporative dry eye [58]. Extrinsic factors include topical medications, use of contact lenses and ocular surface disease such as allergic reactions [59].

Hyperosmolarity, the core mechanism of dry eye disease, leads to ocular damage directly or by triggering inflammation which triggers a so-called vicious cycle of subsequential events, shown in Figure 1-2. Both form of dry eye, evaporative and aqueous deficient dry eye can give rise to tear hyperosmolarity by evaporation. Initiating a cascade of signalling events on the ocular surface leads to the release of inflammatory mediators such as IL-1, IL-17, IL-6, TNF-α. All those mediators all together cause ocular surface cell death and damage to the ocular surface. Along with tear film instability, all those pathways cause amplification and eventually lead to chronic damage and self-perpetuated disease [60].

Normal tear film osmolarity has been agreed as 302.2 ± 8.3 mOsm/L, mild to the moderate dry eye has been diagnosed as 315.0 ± 11.4 mOsm/L and severe dry eye as 336.4 ± 22.3 mOsm/L [61]. Higher osmolarity

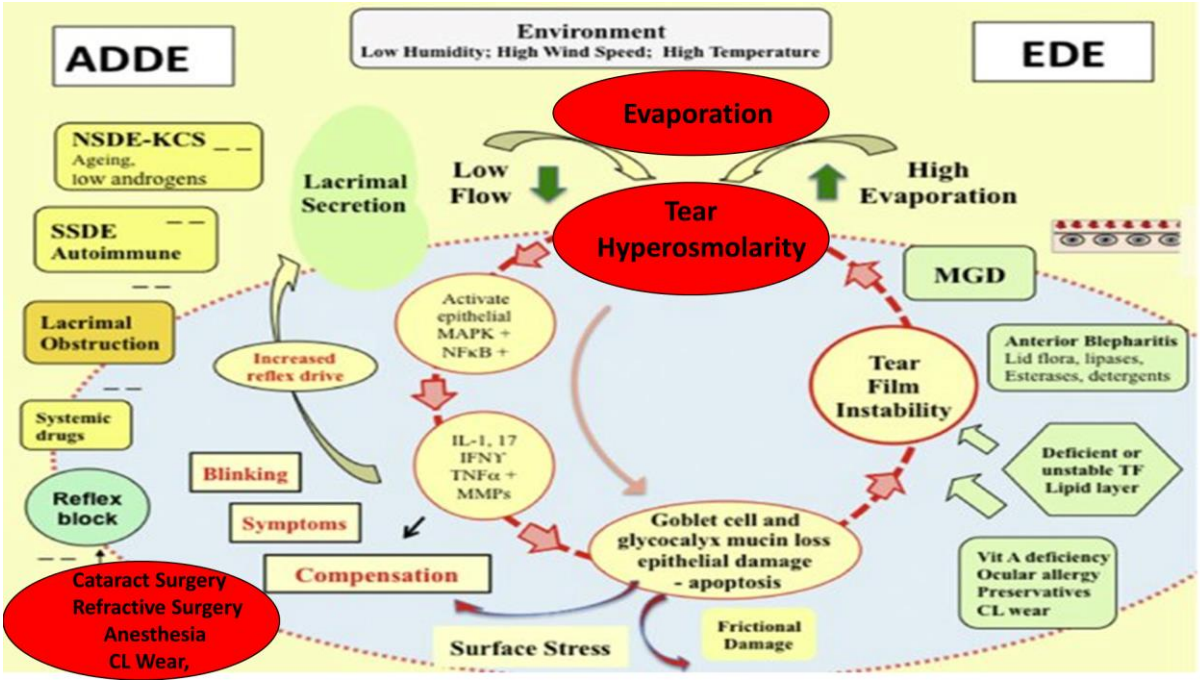

Figure 1-2.Vicious cycle of dry eye. Multifactorial dry eye disease pathogenesis is summarized along with risk factors. The schematic diagram is redrawn by the PhD student.

1.3 Risk Factors

Risk factors for dry eye include the following [62]:

- Increasing age [63–66]
- Female gender [63,64,66–68]
- Hormonal alterations especially postmenopausal oestrogen therapy [67,69,70]
- Diet or deficiency of nutrients [71–74]
- Systemic disease such as diabetes, HIV [75,76]
- Contact lens use [68,77,78]
- Medical treatments like antihistamines or transplant surgeries [79–82]
- Ocular surgery such as cataract surgery, corneal refractive surgery [2,83–85]

1.3.1 Age

Age has been found to be a significant risk factor for the dry eye from large epidemiological studies such as Women Health's Study and Physician's Health particularly after the age of 50 [63,86]. In the aging eye, the reduced blink rates, oxidative stress, polypharmacy, reduced androgen levels will all contribute to likelihood of generation of dry eye disease[87]. Moreover, even though dry eye disease effect on visual acuity is generally considered as mild to moderate, damage on the ocular surface due to dry eye disease in elderly patients can lead to a significant decrease in the quality of life and visual [87].

1.3.2 Sex and Hormonal Alterations

The Woman's Health Study has revealed that the incidence of dry eye in females is almost three times higher than in males [63]. In detail, there is a higher risk (7 %) for women undergoing hormonal changes (menopausal or postmenopausal) to develop dry eye disease compared to women not in the same age range [62,63]. Moreover, women undergoing postmenopausal hormonal therapy are more likely to develop dry eye disease in comparison to women who are not undergoing hormonal therapy [63,69]. From this, it is clear that oestrogen is a critical factor for dry eye disease development and in 2001, Schaumberg and colleagues compared an oestrogen only therapy with a progesterone-and-oestrogen therapy [70]. Their results indicated the oestrogen-only therapy increased the risk of developing dry eye by almost 40 % while the use of progesterone significantly mitigated this effect [70].

1.3.3 Nutrition

Vitamin A and omega-3 deficiency, or a higher ratio of omega-6 compared to omega-3 fatty acids have been shown to significantly increase the risks of dry eye [52]. Moreover, dry eye patients have shown decreased levels of Vitamin D [73,74]. Hence, it has been suggested that Vitamin D could be used as a potential treatment, however, further research is required to explore the safety and efficacy of this solution[88,89].

1.3.4 Systemic Diseases

Diabetes has been shown to increase the risk of dry eye by 15.5 % in children [90] and 54.3 % in a group with a mean age of 54 ± 11 years [75]. In addition, several studies have demonstrated the association of some systemic disease with dry eye diseases such as rheumatoid arthritis [91], fibromyalgia [91,92], systemic lupus erythematosus [93], psoriatic arthritis [94] and chronic hepatitis C [95].

1.3.5 Contact Lens Use

Historically, it has been widely shown that contact lens wearers are more prone to symptoms of ocular dryness and discomfort [77,96]. This may be due to the lowering of the polar lipids combined with an increasing concentration of nonpolar lipids due to greater friction at the ocular surface [19]. Moreover, additional evidence has shown that contact lens wear may lead to a dramatic reduction of healthy meibomian glands [78].

1.3.6 Medical Treatments and Transplants

Long term use of medications such as antihistamines, or corneal transplantation procedures can cause hypersensitivity and/or toxicity make the eye susceptible to dry eye disease [60,97]. Yotnuengnit and colleagues have investigated the severity of the dry eye disease after hematopoietic stem cell transplantation [82]. Out of 28 patients who had autograft, 57% of the patients had a dry eye after the transplantation. Patients who had allograft (48 patients), 50% of the patients were recorded for suffering dry eye after the transplantation.

1.3.7 Ocular Surgery

In the last few decades, several patients undergoing ocular surgery have shown signs of dry eyes [2,84,85,98,99]. Cataract surgery [83,85,100–105], refractive surgery [106–111], glaucoma surgery (trabeculectomy) [112,113] can induce dry eye or worsen the pre-existing dry eye condition in patients. A number of possible factors for dry eye in ophthalmic surgery have been suggested including the use of a speculum [105], the creation of the surgical incisions [101], antiseptic agents [114,115] and light from the operating microscope [105,116–118]. Potential causes have been identified in corneal neural damage [104,110,111,119,120], inflammation of the ocular surface [99,104,121–124] and pre-existing

tear film deficiency [99,100,102,125,126] and tear film osmolarity [102,127]. However, there still remains a limited understanding of the specific mechanisms of these surgical procedures that are triggering dry eye [116,128].

1.4 Cataract Surgery

Cataract is a loss of transparency of the lens in the eye and it is treated by surgically removing the crystalline lens and replacing it with an intraocular lens (IOL). It is one of the most frequently performed surgeries in the world [129] however, it is still not fully understood why cataract surgery may lead to postoperative dry eye in patients [130].

The pre-operative pharmacological agents used for cataract surgery include anaesthetic and pupil-dilating agents. There are various options available for anaesthesia including topical, sub-Tenon's, peribulbar, retrobulbar and general anaesthetics. A local anaesthetic is normally applied topically or by injection prior to cataract surgery [131,132]. The procedure is initiated by preparing the eye with mydriatics and the application of the antiseptic agents. Application of the speculum follows with a drape which is applied to cover the face except for the operated eye. A small corneal incision (usually between 1.5-3 mm) is created to gain access to the anterior chamber [133]. A capsulorhexis is performed, which involves creating an opening to the anterior capsule. Consequently, an injection of fluid under the anterior capsule is performed to hydro-dissect the lens cortex from the capsule and an ultrasound probe is used to break up the lens and remove it in small pieces from the capsular bag (phacoemulsification) [129,134]. Finally, an intraocular lens is implanted using a specifically engineered injector.

More recently, femtosecond laser technology has been applied in some centres to replace some steps of the procedure. More specifically, the laser can be used to create the corneal incisions, the capsulotomy and also to fragment the crystalline lens prior to phacoemulsification [129,135]. More recent research has shown that the clinical outcomes of laser-assisted cataract surgery are not superior to those obtained without the laser [101,136].

The choice of pre- and post-operative medications will depend on the surgeon's preference as well as patient-specific factors such as patient's age, medical and ocular history [137]. A diluted povidone-iodine solution is widely used at the beginning of the procedure in many countries for skin and conjunctival antisepsis [138]. Prophylactic intracameral agents at the end of the procedure, such as cefuroxime or vancomycin, are used to reduce the risk of endophthalmitis, an intraocular sight threatening postoperative infection [139].

Postoperatively, antibiotics, nonsteroidal anti-inflammatory drugs (NSAIDs) and corticosteroids are other agents that may be used prophylactically for days to weeks against postoperative infection and inflammation. While corticosteroids are effective on the inflammatory mediators, NSAIDs act by blocking cyclooxygenase enzymes and prostaglandin synthesis [124,140]. NSAIDs such as diclofenac, ketorolac, bromfenac and nepafenac are applied to reduce pain and inflammation [141,142]. These post-operative agents are most commonly preserved in multi-dose bottles. Repeated use of preserved ophthalmic medications can have an adverse impact on the ocular surface including disruption of the cellular membrane and an increase in corneal epithelial permeability, tear instability with excessive evaporation [143], corneal damage, inflammation and hyperaemia, and eventually reduced vision [143,144].

Dry eye disease can also adversely impact visual outcomes and comfort following cataract surgery [2,84,99,100,130,145–147]. Preoperative tear film quality can have an impact on the accuracy of preoperative measurements such as ocular biometry which is used to calculate intraocular lens (IOL) power to a high degree of accuracy [145]. Dry eye disease could affect these measurements and hence the postoperative refractive outcome [110,145,148]. Therefore, efforts should be made to optimise the ocular surface prior to and after cataract surgery in order to meet the very high patient expectation for refractive predictability.

1.5 Prevalence of Dry Eye After Cataract Surgery

Patients who have undergone cataract surgery may complain of ocular discomfort due to dry eye [130] although the reported prevalence of DED is variable due to the lack of universally agreed diagnostic

criteria. One study focused on the diagnosis of dry eye and its prevalence in a cohort of 50 eyes of 37 patients who underwent cataract surgery [149]. The authors used the Ocular Surface Disease Index (OSDI) questionnaire, corneal and conjunctival fluorescein staining, tear break-up time, Schirmer test and impression cytology for the diagnosis of dry eye at 1 week, 1 month, and 3 months after surgery. It was found that the Schirmer test and tear break-up time was significantly worse after cataract surgery in most of the patients. Dry eye started to be observed at 1 week and peaked at 1 month after the surgery and did not recover even at 3-month time point. Vehof et al. [3] found a strong association between dry eye and cataract surgery with more than 70% prevalence in the female cohort study with a population of 3824 women in the UK diagnosed by structured postal questionnaire. In another study, Cho and Kim [147] studied 70 eyes of 35 patients, who did not experience dry eye prior to surgery. Dry eye indicators such as tear meniscus height, Schirmer test, tear film break-up time, and subjective questionnaires; worsened after cataract surgery. However, follow-up intervals were only 1 to 10 days, making it impossible to comment on possible improvement over time. However, Liu and colleagues demonstrated that 19% of their patient cohort showed a reduced tear break-up time 30 days after surgery [150]. Kasetsuwan and colleagues conducted a study on 92 patients that were not affected by any ocular disease rather than cataract [130]. The incidence of the dry eye diagnosed 7 days postoperatively with TBUT was 68.4%, with Schirmer test it was 12%, with the Oxford Schema Staining it was 58.7% and with the OSDI the prevalence was 10%. The variation observed between these tests is partly due to the sensitivity of the diagnostic tests confirming the difficulty of diagnosing dry eye after cataract surgery [130]. Furthermore, longer follow-up time would be beneficial to investigate the recovery after surgery.

1.6 Aetiology of Dry Eye After Cataract Surgery

The underlying mechanism of DED following cataract surgery could be related to the disruption of corneal nerves [102,151] and corneal epithelium due to surgical incisions [99,152], intraoperative irrigation of the ocular surface [131], light from the operating microscope [116,147,153], elevation of inflammatory mediators in the tear film [2], anaesthetic agents [154] and postoperative eye drops (active agents and/or preservatives) [130,147].

1.6.1 Corneal Incision

The cornea has the highest density of sensory nerve endings in the human body, hence any corneal incision would impair the sensitivity of the cornea [99,155]. Transection of corneal nerves can lead to impaired epithelial wound healing, higher epithelial permeability, reduced epithelial activity and the destruction of cell cytoskeletons, ultimately damaging the overall corneal metabolic function [106,108]. Corneal sensitivity reduction is also observed affecting the tear film production that leads to aqueous deficient dry eye [100,119,151,156]. Fortunately, corneal cells have the capacity to restore tissue homeostasis following surgical incisions, depending on the size of the incision. In particular, smaller incisions have been shown to reduce corneal nerve disruption and its deleterious effects compared to larger incisions [127].

1.6.2 Inflammation

Cataract surgery has already shown the potential of inducing inflammation at the ocular surface [99,122,157]. Inflammation can be associated with both types of dry eye (evaporative and aqueous deficient dry eye), which is therefore considered as the core mechanism of dry eye [158,159]. It can induce many metabolic processes such as inflammatory cell migration, elevation of inflammatory cytokines, chemokines and different enzymes [160]. Inflammation markers have been also shown to increase in dry eye patients [161–164]. In addition, inflammation marker differences before and after cataract surgery were recently explored. Tumour necrosis factor alpha (TNF-α), vascular endothelial growth factor (VEGF) and different interleukin (IL) cytokines and chemokine; IL-1, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10 were found to be significantly increased in tear samples of dry eye patients [55,161,162,165–167]. In 1991, Malecaze and his colleagues found an increase in IL-6 levels after cataract surgery at day three in 12 patients' aqueous humour compared to preoperatively [167]. On the other hand, they did not find any elevation in the patient's serum which indicates the local ocular production by fibroblasts, T cells and epithelial cells [167]. One of the recent studies carried out by Park's group examined inflammation markers from 34 patients who were undergoing phacoemulsification cataract surgery in order to observe the difference before and after surgery [2].

Patients with and without dry eye were compared before and after cataract surgery. They found that IL-1β, IL-6, IL-8 and TNF-α were higher in tear samples of patients with preoperative dry eye at day 1, and started to decrease at month 1 and 2 within 18 patients [2]. Changes in IL-6 levels were found to be significantly correlated with dry eye parameters such as corneal staining and tear break-up time. Even though the sample size was relatively small but powered to detect the difference, significant changes were observed in the inflammation markers levels before and after the surgery conditions [2].

1.6.3 Light from Operating Microscope

Operating microscopes are necessary for modern surgical facilities and very important factor for the success of the most of the complex microsurgeries in medicine today. The chosen light intensity used by ophthalmologists were recorded mostly between 27,000 to 40,000 from 20cm [116]. Most of the operating microscopes for ophthalmic surgeries was designed to minimize the risk damage to the eye. Filter use, for that reason, is very common to limit the UV exposure (180 nm -400 nm) to reduce the photochemical toxicity and infrared exposure (780 nm and above) to protect from thermal injury.

The light of the operating microscope used during the surgery might be important to take into consideration as a cause of dry eye [116,153,168]. There have been several studies about the photic effect of the operating light microscope on the retina, however, the influence of the effect on the ocular surface is not well understood even though it is the first barrier for light to penetrate the eye. Few studies have explored the effect of the operating light on the ocular surface [116,130,147,169–171] therefore, more research is required to understand the possible effect of light on the ocular surface. Michael and Wegener in 2004 determined the amount of time needed for the operating light to become toxic for both retina and cornea [170]. They suggested the use of UV filters for ensuring corneal and retinal safety. Five years later, Cho and Kim [147] assessed dry eye parameters postoperatively at day 1 with microscopic exposure time, and found a significant correlation between longer exposure times and increased dryness symptoms and reduced tear break up time. There was a lack of information about the duration of the light exposure during surgery, therefore it was not possible to compare this study with relevant literature. In 2014, Hwang and his colleagues studied the

phototoxicity of an operating microscope on the ocular surface of rabbits [116]. They found reduced aqueous tear production, weakened corneal and conjunctival epithelial cells, decreased goblet cell density and increased IL-1β levels with 30 minutes of exposure to the light, assessed at 1 day to 5 days. Their results suggested that exposure to the operating microscope light could be a potential risk factor for the development of dry eye [116]. However, there were no data on possible longer-term effects. Moreover, rabbit eyes are known to be biochemically and physiologically different from human eyes [172]. They are characterised by a thinner cornea, a higher threshold to pain, lower tear yielding and reduced ocular surface sensitivity, which are important disadvantages to take into consideration when using rabbit models for studying dry eye [173,174].

A recent study by Kohli and his colleagues found a significant correlation between dry eye parameters (tear break up time, corneal fluorescein staining and Schirmer test) and duration of the operation, up to 6 weeks after surgery [175]. However, they did not find any correlation between goblet cell density and the duration of the operating microscope exposure. This may possibly be due to the limited sample size of 50 eyes which was also mentioned as one of their limitation and their short follow-up design. In contrast, Oh et al. [117] found a significant correlation between goblet cell density and operating microscope exposure time in a study of 30 eyes. In addition, they observed that goblet cell density did not recover up to 3 months after surgery although the tear film break-up time returned to preoperative levels after only one month. Lastly, Sahu and colleagues did not find any correlation between dry eye diagnostic test values and the microscope light exposure time during phacoemulsification [176]. This might be also due to the different type of keratome used during cataract surgery to create an incision, in which Sahu and colleagues used 3.2 mm instead of 2.8 mm incision size as the other studies discussed above.

Until now, there have been only a few studies in vitro or in vivo study which has explored the potential phototoxicity on ocular surface by the operating microscope other than mentioned above. These contradictory results highlight the need for the development of in vitro models along with more clinical investigations that could allow scientists to reproducibly study the potential phototoxic effect of surgical microscope light and its correlation with dry eye.

1.6.4 UV Irradiation

During cataract surgery, the ocular surface is directly exposed to the operating microscope light for a period of time ranging from around 10 minutes or so to over an hour [177] however, there are only a few studies giving attention to UV radiation from operating microscope. In the last decade, few scientists around the world have focussed their attention on how UV radiation could cause damage to the ocular surface from operating microscope exposure [116].

When Hwang et al. [116] found ocular surface damage and tear film instability with the operating microscope on rabbits' eye, they hypothesized that phototoxicity could be due to the UV-B radiance emitted by the operating microscope. Although they did not display their microscope spectral data for UV levels which could have been useful to compare for future studies, they observed damage with light exposure.

It is already known that the cornea has been shown to absorb UV radiation below 295nm [178] and the tear film has been shown to absorb wavelengths between 280nm to 315nm [179]. However, cumulative UV exposure has been shown to damage the cornea [180] and conjunctiva [181]. Clinically, direct UV exposure has been demonstrated to lead to photokeratitis and conjunctival disorders such as pterygia [182]. Therefore, it is important to understand the effect of UV radiation emitted from the operating microscope.

In addition, there is a body of growing evidence exploring environmental UV exposure and its association with dry eye [179,183,184]. Studies came to quite contradictory conclusions [60,185,186] and hence, UV light has not been stated as a risk factor for dry eye in the TFOS DEWS II Report [187]. In particular, the UV exposure on outdoor workers has been investigated by assessing tear film instability and dry eye prevalence in a large scale study in Indonesia [188]. The authors did not find any correlation between dry eye prevalence and time spent outside, although further research in a more controlled environmental fashion in terms of temperature, oxygen level is required. However, Feretis and his colleagues found the higher prevalence of ocular surface damage in fisherman compared to a

group of monks in Athens although the paper lacks the information about the description of ocular damage [186].

UV radiation is classified as a risk factor for pterygia, a degenerative disease caused by excessive growth of bulbar conjunctiva that affects vision and leads to tear film instability and consequently dry eye [185,189]. Pterygium has been studied in vitro and direct UV exposure has been shown to induce inflammatory processes and angiogenesis in these cells [190]. UV radiation was extensively studied in vitro using conjunctival epithelial cells and fibroblasts to show the association between dry eye and pterygia. In 2009, Viiri and colleagues studied the response of human corneal and conjunctival epithelial cell lines to UV-B radiation of 153 mJ/cm² [191]. They found elevation of IL-6 and IL-8 and reduced viability of conjunctival and corneal cells. Buron et al. [181] found UV radiation of 30 J/m² caused caspase-dependent cell death in the conjunctival cell line. Moreover, Schmut and his colleagues found conjunctival fibroblast viability was reduced by 28% when exposed to 30 mj/cm² of UVB irradiation as one of the possible risk factors for a dry eye [192]. These studies proved that UV radiation induces apoptosis and acute inflammation which can contribute to ocular surface disease such as dry eye. However, the association between UV radiation and dry eye is still unclear due to the lack of consensus and variation in different in vitro models used and the lack of clinical evidence.

Dry eye disease, due to less tear production and/or change in the flow of tears, could directly affect the UV-absorbing protective feature by the tear film [179]. The lack of appropriate protection can potentially make the ocular surface more susceptible to UV radiation. This might be a reason why dry eye patients suffer from photosensitivity [187], preoperative dry eye patients could be more sensitive to UV radiation from the operating microscope, compared to healthy eyes. However, it appears no study has explored this.

1.6.5 Topical Medications

Topical medications could also lead to dry eye due to the presence of preservatives in multi-dose eye drops [143]. Studies have shown that preservative-free eye drops provide better results in terms of preventing ocular inflammation and reducing the risk of dry eye after surgery [193,194]. For example,

Jee and colleagues [194] evaluated the use of 0.1% sodium hyaluronate and 0.1% fluorometholone preservative-free eye drops compared to the preserved version of these agents after cataract surgery. They found significant improvement in postoperative signs and symptoms in terms of inflammation level, tear break-up time, with ocular surface staining in the preservative-free version of these agents post-surgery. There is a lack of longer-term follow-up studies to determine whether using non-preserved eye drops might be better in terms of toxicity. However, there is naturally a risk of contamination of using non-preserved agents compared to preserved eye drops if they are not single dose unless there are other preventative mechanisms against contamination in place. The single dose versions of unpreserved eye drops are usually more expensive than preserved eye drops and may be difficult to instil depending on the delivery systems sometimes. On the contrary, multi-dose eye drops could be used with preservative filtration and adsorption on a membrane, or they could be applied with a valve system prevent possible contamination [143]**.**

In terms of antiseptic choice, over the years, several antiseptics have been used to prevent endophthalmitis postoperatively [114]. One of the most frequently used antiseptics before cataract surgery is povidone iodine (PVI), which is used for intracameral purposes [195] and for skin disinfection [196]. It is mainly used at a concentration ranging from 1% to 5% to disinfect the conjunctiva [197]. Although the European Society of Cataract and Refractive Surgery advise a 3-minute instillation of 5-10% PVI before cataract surgery [139], several studies have reported on the toxicity of PVI suggesting it is a cause of postoperative dry eye [198,199].

The concentration from 5% and 2.5% PVI instillation to the conjunctival sac caused severe corneal epithelial damage on 12 rabbits while 1% and 2% of PVI led to corneal oedema with 30 minutes exposure [200]. The effect of 30-minute exposure of 0.0125% PVI on human corneal epithelial cell line was shown to reduce viability by 30% [199]. In 2014, Shibata and his colleagues investigated the corneal epithelial cell response after 30 seconds of exposure to PVI and found cytotoxicity even at PVI concentrations as low as 0.5% [201]. Moreover, PVI was found to be able to penetrate inside the cornea, with the immersion of human donor eyes exposed to 0.25% concentration for 2 minutes since corneal fibroblasts were also found to be adversely affected in terms of their viability [202]. The

immersion of human donor eyes into PVI does not closely replicate what happens in vivo before surgery making it complex to apply the results to the in vivo condition, however it proves the toxicity can affect the internal tissues of the eye [202].

Concentration of 0.0125% PVI was found to be cytotoxic to corneal epithelial cell line by Yanai et al. [199] with 5 to 30 minutes exposure. Toxicity was found to be increased with rising concentration and duration. This study also explored contact lens disinfection solutions as some of the solutions contains antiseptic agents like PVI. Knowing the PVI concentrations that avoid toxicity on the ocular surface will also help to find the balance in disinfection solution with antimicrobial activity and biocompatibility.

Finally, the toxicity of PVI has mostly been studied on the cornea, while very few in vitro models explore the effect of PVI on the conjunctiva. For example, Bataille and his colleagues investigated the effects of 5% PVI exposure for 3 minutes on a conjunctival cell line and found that the 'preferred' concentration of 5% resulted in high toxicity [203].

In summary, variation in results between the studies is due to the fact that they used different PVI concentrations with different exposure times. This is because of the lack of a validated in vitro model of the ocular surface, therefore most of the studies use individual models making it difficult to draw conclusions from the varying results.

In conclusion, a better understanding of the effect of PVI exposure to the ocular surface will help to find the correct balance between antimicrobial activity and biocompatibility for applications such as cataract surgery and in disinfection solutions.

1.6.6 Speculum Use

It has been speculated that possible contributory factors in dry eye after cataract surgery could be due to the use of a lid speculum [2,105]. Decreased blinking rate can lead to lid dysfunction due to the restricted release of meibum which can result in meibomian gland dysfunction along with reduced corneal sensation after surgery. Moon et al. [105] investigated the speculum effect on dry eye parameters with 58 eyes who underwent cataract surgery. Significant disruption was observed with the use of speculum by conjunctival staining, tear break up time, conjunctivochalasis and OSDI

questionnaire up to day 7 postoperatively. Even though the symptoms returned back to baseline levels at 1 month postoperatively, the use of a speculum should be taken into account when exploring the dry eye after cataract surgery. Further follow-up and more clinical studies are required to clarify the effect of speculum on the ocular surface.

1.7 Dry Eye Treatment

Although this thesis scope is not the treatment, some of the available treatment options are briefly summarised. More details can be consulted to DEWS II [204]

Artificial tears are frequently used to alleviate dry eye symptoms [205]. Ocular lubricants and gels are the most common types of artificial tears present in the market, especially those with Hyaluronic Acid (Hy-A). The latter are frequently prescribed due to their capacity to hold more water and the efficient hydration of the ocular surface [205,206]. Artificial tears containing Carboxymethylcellulose (CMC), are commonly used for their ability to bind to disrupted areas of the epithelial surface, consequently, increasing the ocular residence time [207]. However, all tear supplements are not a long term solution, for dry eye and need to be applied several times a day.

Punctal plugs are inserted into the puncta in order to prevent drainage of tears. However, complications including discomfort and possible excessive tear flow or extrusion of plugs are very common with them [208].

Thermal therapy for meibomian gland dysfunction can be performed using warm compresses, EyeBags (The EyeBag®, West Yorkshire, UK), Blephsteam (Thea, Newcastle-Under-Lyme, UK) or Lipiflow (TearScience Inc., Morrisville, NC). The purpose of all types of thermal therapy is to induce and/or increase the rate of lipid secretion from meibomian glands to the tear film.

Since inflammation is one of the key factors of dry eye, anti-inflammatory agents are also used as a treatment for dry eye. Cyclosporine, corticosteroids, omega-3, omega-6 supplements and monoclonal antibodies have been used to reduce inflammation in dry eye patients [57]. Among them, cyclosporine is the most common anti-inflammatory agent, and it works by inhibiting T-cells to

relieve dry eye symptoms. However, there are several discussions on the side effects of this anti-inflammatory agent, such as neural toxicity, ocular hypertension and infection [209].

Currently, there are no long term treatments for dry eye at the moment although research is constantly testing potential new therapies to address the pathology [204].

1.8 In vitro models of Dry Eye

1.8.1 Two Dimensional Models

Hyperosmolar stress has been used to study dry eye in vitro due to its simplicity and its key role in the pathogenesis of ocular surface damage in dry eye disease. Hyperosmolar stress was found to cause corneal and conjunctival epithelial inflammation [210,211], the elevation of pro-inflammatory cytokines [212,213], cell death [214], and apoptosis [210]. Therefore, it has been considered as a suitable way of mimicking dry eye pathology in vitro. Corneal, conjunctival epithelial, limbal stem cells and cell lines have been extensively used in in vitro with hyperosmolar stress to observe the behaviour of the cells and molecular pathways under that stress, not only to understand the pathology and also testing new therapies for treatment. One of the pioneer studies conducted in 2006, Li and colleagues [213] studied limbal epithelial cells for 24 hours in hyperosmolar conditions ranging from 312 mOsm to 500 mOsm by adding 0, 30, 50, 70 and 90 mM NaCl to their basal medium. This is one of the first studies in vitro elucidating the association between inflammation and hyperosmolar stress on ocular surface cells [213]. IL-8, IL-1β, IL-6 and TNF-α were found to be elevated for the first time correspondent to concentration-dependent hyperosmolar stress by corneal and limbal epithelial cells. Lower than 370 mOsm osmolar medium did not significantly alter the production of the cytokines, which is most likely due to the resistance to the hyperosmolarity in vitro conditions by the cells [213]. Another study by Igarashi and colleagues observed corneal epithelial cells for 10 minutes and 24 hours under hyperosmolar medium (from 400 to 1000 mOsm/kg) and found an increase in IL-6 levels with higher than 600 mOsm/kg medium conditions even after just 10 minutes, confirming the association with inflammation and hyperosmolar stress [215].

Although most of the literature investigating dry eye disease in vitro focuses on the cornea, a growing body of literature has started to recognize the importance of conjunctival research for dry eye [165,168,191,214,216,217]. Human HeLa modified conjunctiva-derived epithelial cell lines were used in a dry eye in vitro model to investigate the pro-inflammatory intracellular mechanism with hyperosmolarity stress (500 mOsm) [218]. Conjunctival cells were found to increase the level of CCL2 secretion which is a chemoattractant protein for monocytes to migrate to the inflammation site, which is a sign of the inflammatory pathway to target for future therapies. They used higher hyperosmolar stress between 340 mOsm (as a control)- 600 mOsm for 24 hours to simulate dry eye disease. They did not find any difference in viability at 24 hours less than 500 mOsm/kg. Even though their control medium osmolarity was higher than the healthy tear film osmolarity condition (302.2 ± 8.3 mOsm), cellular health was not affected by this amount of difference. This is also another confirmation of the in vitro resistance of the cells to hyperosmolar stress compared to in vivo.

Clouzeau et al. [214] combined hyperosmolarity and benzalkonium chloride as two stressors applied to conjunctival epithelial cells derived from Wong Kilbourne (Chang conjunctiva-derived cell line) cultured under NaCl-induced hyperosmolar conditions (400–425–500 mOsM) for 24 and 48 hours. They found ccytotoxic effects; cell death, chromatin condensation, F actin disorganization with the hyperosmolar stress in the line with corneal cells in a concentration-dependent manner.

A recent study carried out by Marek et al. [168] cultured the conjunctival epithelial cell line under hyperosmolar stress (320-460 mOsm) and investigated the effect of blue light exposure on those cells along with hyperosmolar stress. This was the first time light exposure and hyperosmolar stress have been combined in vitro to observe whether dry eye patients are more sensitive to blue light. They found a greater increase in IL-6 levels, reactive oxygen species, and a decrease in viability with hyperosmolar stress in conjunctival cells. This is the first study that combined these two stressors and indicated that cells under hyperosmolar stress can be more affected by phototoxicity. Until now, there have been no studies investigating conjunctival primary cells within hyperosmolar stress along with the light exposure to relate to dry eye disease.

The main common limitation of these studies is using cell lines which can potentially differ in behaviour from primary cells in terms of their native functions and responses to stimuli [219–221] even though they have the advantage of repeatability [222]. Unfortunately, there are only a few in vitro studies which used primary cells in dry eye disease [40,223,224]. Versura et al., [211] found the correlation between 24-hours of hyperosmolar stress on human conjunctival epithelial cells with expression of human leukocyte antigen (HLA-DR, a pro-inflammatory marker which has a role in initiating the inflammation response). This study confirmed that hyperosmolar stress is able to trigger and perpetuate the inflammation cycle by using the primary cells. They selected the hyperosmolarity stress as 350 and 400 mOsm/L since they did not find any difference between HLA-DR levels with lower hyperosmolar stress, which is in accordance with previous findings with the corneal cells [225,226].

Higher hyperosmolar stress than the typical in vivo conditions has been used to simulate dry eye in vitro due to the nature of the in vitro models. This is because the cells show higher resistance to high osmolarity due to adaptation carried out by membrane cotransporter activity in vitro [227]. Furthermore, most of the in vitro studies expose the cells to hyperosmolar stress for a very limited time compared to in vivo. Therefore, the higher osmolarity is usually preferred to simulate the longer-term nature of the dry eye condition. Another explanation for using higher osmolarity is that tear osmolarity is not stable throughout the ocular surface. The lower tear meniscus osmolarity level differs from the pre-corneal surface. Liu et al [228] reported that hyperosmolar drops with higher than 800 mOsm/kg gave patients a burning sensation which is one of the dry eye symptoms. They also investigated that drops with 600 mOsm/kg osmolarity are able to induce inflammation by activation of the mitogen-activated protein kinase (MAPK) pathway which has an important function in signal transduction for cell proliferation and cell death. They concluded that the tear film of dry eye patients may transiently spike leading to inflammation in the cornea. It is also speculated that the osmolarity of the tear film may not be distributed equally over the ocular surface [215].

Another consequence of dry eye pathology on the ocular surface is delayed wound healing which is also important when studying the effect on ocular surgery [153]. It has been known that dry eye can result in impaired wound healing after cataract and refractive surgery [109]. Therefore, there has been

a great interest in recovery from damage due to being self-perpetuating disease. The scratch assay has been used to study a simplified wound healing response in vitro [153,168,229]. The scratch assay has been used on corneal epithelial cells [230–232], stromal cells [164] predominantly, and on conjunctival cells [233] to study the effect of different dry eye treatments [234] and growth factors [230].

A recent study carried out by Wang and his colleagues performed a manual scraping on mouse corneal epithelium and monitored the wound healing with different stressors [235]. A significantly delayed healing response was observed when the mouse eyes were exposed to hyperosmolar stress or IL-1β or TNF-α. Hyperosmolar stress was found to cause more delay in wound healing than exposure to IL-1β or TNF-α alone. Although this study established the two core mechanisms of dry eye; hyperosmolarity and inflammation in vivo and in vitro, it lacks the justification of some of the important aspects. The exposure to hyperosmolar and inflammation stressors lasted for 8 days in which the authors failed to fully define the culture time difference compared to previous in vitro models since it is longer than the usual culture times in vitro. Moreover, no information about the status of the cellular health in terms of their viability (6 well plate) was provided, since the cells were not designed to culture long term on tissue culture plastic [236,237].

As mentioned above, there are few conjunctival in vitro models for exploring dry eye disease and until now there has been no study investigating conjunctival fibroblasts under dry eye conditions using hyperosmolar stress.

1.8.2 Three-Dimensional Models

In the last decades, many experimental models have been used to help understanding dry eye disease [213,238–240]. In vitro models offer a simplified version of a diseased system to study risk factors and/or analyse the cytotoxic effect of new treatments [241]. Most of the experimental models have been developed using corneal, conjunctival cells in three-dimensional models. For example, Meloni and colleagues cultured human corneal epithelial tissues for up to 72 hours in a controlled environment with humidity lower that of 40% and temperature of 40 °C [242]. Another dry eye model was developed using a whole rabbit cornea cultured for 21 days in order to study corneal re-

epithelization and wound healing [238]. Barabino and colleagues used a reconstructed human corneal epithelial model using a human corneal epithelial cell line from EPISKIN laboratories (Lyon, France) in a dry-hyperosmolar condition (at less than 40% humidity, 40°C temperature) [239]. The common limitation of both culture models is lacking the long term culture of the cells within the model.

The recent development of a three-dimensional conjunctival model study including the epithelial layer fibroblasts and goblet cells in a fibrin scaffold together. The epithelial cells were cultured on top of the goblet and fibroblasts in the fibrin scaffold [243]. In order to simulate dry eye conditions, the construct was left for 2 hours without media as a partial desiccating condition. Cell morphology, inflammation cytokines such as IL-6 were found when the simulation of dry eye was performed although the goblet cells did not survive until the end of culture time which is one of the limitations of the study.

There are also more simplified three-dimensional models (3D) with scaffolds, instead of using plastic to culture cells in vitro in order to overcome long-term culture problems. There are currently a few models constructed to study the conjunctiva as a whole tissue [243–245]. Collagen, as the most abundant component in the extracellular matrix of the ocular cells particularly conjunctival stroma, has been used as a potential scaffold to study different ocular surface disease pathology [45,246]. Gater and her colleagues developed a 3D model with porcine conjunctival fibroblasts for investigating and finding new therapies for fibrosis, which is a common reason for failure in glaucoma surgery, such as trabeculectomy [244]. They used collagen hydrogel to mimic the matrix environment in order to better simulate the in vivo environment. Even though conjunctival fibroblasts were important for the ocular surface inflammation response, which is the main core mechanism of dry eye, no study has investigated those cells within a three-dimensional environment for exploring dry eye pathology.

1.8.3 Cell Source

Due to the restricted availability of human eyes for research purposes, cells derived from animals have been preferred for use in in vitro research. In vitro models constructed to study dry eye have been mostly based on rabbit or mice. Those animals have been preferred both in vitro and in vivo research to investigate the pathology of dry eye due to the availability of transgenic strains and the ease of

handling of the animal. Porcine eyes have been used in view of the similarity to human eyes in terms of their tear film structure [173,247,248]. There is also evidence that porcine and human corneal cells show similar phenotype and molecular responses [249]. Being a waste product from the abattoir, porcine eyes provide a reliable, cost-effective and high-quality source of tissue and cells for in vitro research for ophthalmology [173]. Therefore, in this thesis, porcine conjunctival cells were obtained from freshly taken eyes from a local abattoir.

1.9 Conclusions and

Dry eye has been affecting hundreds of millions of people in the world, including 40 million people in the US alone [31]. Cataract surgery, one of the most commonly performed surgeries in the world, is one of the risk factors for dry eye. Patients' expectations after cataract surgery are typically very high, in terms of their vision and comfort, and postoperative dry eye can significantly affect patient satisfaction. A review of the scientific literature on post-operative dry eye following cataract surgery reveals there are several gaps in the current knowledge about why dry eye signs and symptoms develop and/or are exacerbated.

1.10 Aims of the Project

First of all, most of the literature has been dedicated to understanding the relation between dry eye and the corneal response after cataract surgery, perhaps due to the location of the incision. However, the conjunctiva plays a pivotal role in terms of maintaining the homeostasis of the ocular surface after surgery. Therefore, there is a need for novel conjunctival in vitro models that could elucidate the mechanisms behind dry eye disease after cataract surgery. In this thesis, a conjunctival in vitro model was developed to study the cellular response to the different steps of cataract surgery.

Secondly, the role of the conjunctival stroma in ocular surface diseases has not been yet extensively studied even though the conjunctival stroma plays a significant role in terms of inflammation, fibrosis and dry eye. In fact, conjunctival epithelial cells can be damaged in severe dry eye conditions, which can allow hyperosmolar tears to reach the conjunctival stroma and make direct contact with the conjunctival fibroblasts. So far, there have been no published in vitro dry eye studies investigating the

effect of dry eye on conjunctival fibroblasts. In this thesis, conjunctival fibroblasts were exposed to different hyperosmolar stress conditions to further elucidate the conjunctival role in dry eye disease.

Thirdly, light exposure and antiseptic agents have been shown to potentially be implicated in the development of dry eye seen after cataract surgery. Both the visible and UV light generated by the operating microscope on the ocular surface has already been studied clinically [116,171,176,250]. However, due to the inconsistency of these studies, light exposure is still not recognised as a dry eye risk factor in some of the published literature [187]. In addition, there have been no in vitro studies establishing the direct impact of light exposure on conjunctival cells cultured in dry eye conditions. One of the aims of this thesis is to investigate how light from the operating microscope could cause impairment of conjunctival cells at the cellular level. Therefore, in this thesis, a conjunctival in vitro dry eye model has been used to investigate the effect of surgical light exposure on the ocular surface.

Lastly, although the effects of PVI on the ocular surface have been studied [199–201], there is little published research focused on the conjunctival tissue [251–253]. Furthermore, there is an inconsistency regarding the PVI concentrations and exposure times used, consequently creating a need for a validated in vitro model to study PVI toxicity [197]. To fill this knowledge gap, this thesis also investigated the effects of PVI on the ocular surface for different concentrations and exposure times.

All the in vitro models developed in this thesis are based on porcine cells because they are not only very similar to human cells anatomically, but they are also a readily available waste product of the food industry [153,173].

In summary, the purpose of this research is to broaden the cellular level of the understanding and raise awareness of the use of the operating microscope and the PVI not only during cataract surgery but also for any ocular surgery using those procedures. Therefore, this research has a broader impact on a variety of ocular surgeries since the postoperative dry eye is a common problem worldwide. This research aims to provide insight into these aspects of surgery and will improve the knowledge in literature about the risk factor for dry eye after cataract surgery. Moreover, it will hopefully make surgeons more cautious too about using these aspects so that patient's satisfaction can be met.

CHAPTER 2: MATERIALS AND METHODS

2 MATERIALS AND METHODS

Overview

This chapter outlines the general materials, methods, software and instruments used throughout the thesis. Each chapter will include any alterations in the procedures that differ from this chapter, in the methods.

2.1 Materials and Instruments

Chemicals, consumables and instruments are listed in Table 2-1. The catalogue numbers were also provided where possible along with the supplier name.

Materials Name	**Supplier**	**Catalogue Number**
10 ml pipettes	Costar, SLS, UK	4101
1000 µl pipette tips	Fisherbrand, UK	11568442

12-well adhesive plates	Costar, SLS, UK	3513
15 Ml Tubes	Costar, SLS, UK	430791
20 µl pipette tips	Fisherbrand, UK	10527014
200 µl pipette tips	Fisherbrand, UK	11923446
24-well adhesive plate	Costar, SLS, UK	3526
25 ml pipettes	Costar, SLS, UK	4489
48 well plate	Costar, SLS, UK	3548
5 ml pipettes	Costar, SLS, UK	4051
50 ml pipettes	Costar, SLS, UK	4490
6-well adhesive plate	Sigma-Aldrich, UK	CLS3516-50EA
96-well adhesive plate, black with clear bottom	Sigma-Aldrich, UK	CLS3603-48EA
96-well adhesive plate, clear bottom, white	Sigma-Aldrich, UK	CLS3595-50EA
Annexin V binding buffer	Biotium, UK	99902
AnnexinV-CF488A conjugate	Biotium, UK	29005
Aqua Stabil	Julabo, Fisher, UK	
Autoclave	Thermo Fisher Scientific, UK	
Bijous	SLS, UK	SLS7524
Blocking Buffer	Biolegend, UK	927503
Bovine Serum Albumin	Sigma-Aldrich, UK	A3608-100G
Cell Staining Buffer	Biolegend, UK	420201
Centrifuge	Hettich, UK	Rotina 420
Collagen hydrogel, rat tail type I	Corning, UK	354236
Confocal Microscope	Leica, UK	
Copper Sulfate	VWR, UK	23165.298
DAPI	Sigma-Aldrich, UK	D9542-1MG

Dextran	Sigma-Aldrich, UK	31392-50G
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, UK	D2650-100ML
DMEM low glucose (powder)	Sigma-Aldrich, UK	D5523
DMEM Low Glucose	Thermo Fisher Scientific, UK	12320032
Ethanol	Thermo Fisher Scientific, UK	E/0650DF/17
Falcons (50ml)	Costar, UK	430290
Filter paper	Appleton Woods, UK	FP027
Fluorescence Microscope	Leica, UK	
Foetal Bovine Serum	Sigma-Aldrich, UK	F7524-500ML
Formalin	Sigma-Aldrich, UK	HT5012-60ML
Glass Slides	Thermo Fisher Scientific, UK	8037/1
Hand held digital camera	Canon, UK	SX540
Hoechst 33342, 500 µg/m	Chemometec, Denmark	910-3015
Hydrochloric Acid (HCL)	Sigma-Aldrich, UK	318949
Image J Software	Java, US	
Incubator	Thermo Fisher Scientific, UK	HeraCell 150I
Intracellular Staining Perm Wash Buffer	Biolegend, UK	421002
Isopropanol	Thermo Fisher Scientific, UK	P7500/17
Laminar Flow	Thermo Fisher Scientific, UK	HerasafeKS

L-Glutamine	Lonza, UK	LZBE17-605E
Light Microscope	EVOS, Thermo Fisher Scientific, UK	
Light Microscope (for the light source)	Motic, UK	AE200
Live-Dead Viability Kit	Invitrogen, UK	L3224
Lux Meter	Testboy, Germany	TV335
Methanol	Thermo Fisher Scientific, UK	M/4000/15
Micro-Centrifuge Tubes	Thermo Fisher Scientific, UK	FB74023
MTT Formazan	Sigma-Aldrich, UK	M2128
NC-Slide A2	Chemometec, Denmark	942-0001
Nucleocounter	Chemometec, Denmark	NC-3000
Operating Microscope	Zeiss, UK	
Osmometer	Gonotec GmbH, Berlin, Germany	
Penicillin-Streptomycin	Lonza, UK	LZDE17-602E
Petri Dishes (Tissue Culture Treated)-35 mm	Costar, UK	430165
Petri Dishes (Tissue Culture Treated)-60 mm	Costar, UK	430166
Ph meter	Mettler Toledo, UK	5easy
Phalloidin	Invitrogen, UK	A12379
Phosphate Buffer Saline Solution (PBS)	Sigma-Aldrich, UK	D8537-500ML

Pipette gun	Thermo Fisher Scientific, UK	
Plate Reader (Absorbance)	Thermo Fisher Scientific, UK	Multiskan GO
Plate Reader (Flourescence)	Molecular Devices, UK	Spectra Max Gemini EM
Povidone Iodine for skin	Nex Iodio, UK	
Povidone Iodine for ophthalmology	Minims, UK	
Presto Blue Cell Viability Reagent	Invitrogen, UK	A13261
Propidium Iodide, 500 µg/ml.	Chemometec, Denmark	910-3016
PTFE	RS Component, UK	752-600
Sodium Bicarbonate	Sigma-Aldrich, UK	S5761-500G
Sodium Chloride	Sigma-Aldrich, UK	S5886-500G
Sodium Hydroxide	Sigma-Aldrich, UK	8045-1KG
Sodium Pyruvate	Sigma-Aldrich, UK	
Sterile Blades No:11	VWR, UK	233-0024
Sterile Blades No:24	VWR, UK	233-0030
Tissue Culture polystyrene (TCP) T75 flasks	SLS, UK	430641U
Triton X	Sigma-Aldrich, UK	T8787-250ML
Trypsin	SLS, UK	LZBE17-161F
Tween-20	Sigma-Aldrich, UK	P1379-100ml
Via-1 Cassettes	Chemometec, Germany	941-0012
Vimentin Antibody	Sigma-Aldrich, UK	AB1620
Virkon	SLS, UK	CLE1552SP

Water Bath	Grant, UK	

Table 2-1. The materials were listed with the details of the company and its catalogue number.

2.2 Methods

2.2.1 Explant Dissection

Porcine conjunctival fibroblasts were used throughout this thesis. Porcine eyes were freshly (within one hour of slaughtering) taken from a local abattoir by trained personnel of Aston University that selectively collected fresh tissue before the scalding procedure. Collected eyes were transferred in the supplemented Dulbecco's modified Eagle's medium (DMEM) supplemented with 4% foetal bovine serum (FBS), 1% penicillin/ streptomycin, 1% L-glutamine (LG) and 0.2mg/ml Dextran (Mw ~ 250kDa).

Fresh eyes were taken to the cell culture lab within 3-5 hours after enucleation Figure 2-1. The dissection began with a repeated rinsing step with phosphate buffered saline (PBS) to remove any debris and blood around the eye. With the help of dissection tools such as forceps and a scalpel with sterile blades, the excess fat around the ocular surface was removed carefully in order to enable easier access to the conjunctiva.

The bulbar conjunctiva was cut as close as possible to the peripheral cornea. The samples of bulbar conjunctiva tissues were cut into smaller pieces from 5mm up to 50mm in size. The pieces of conjunctiva were collected and rinsed three times with 3% Penicillin/Streptomycin in PBS to disinfect the tissues before the culture. The tissues were transferred to the tissue culture polystyrene (TCP) with different volume such as T25-T75 flasks depending on the tissue number with the bent dissecting needle. Prior to transferring to the flasks, the tissue on the tip of the needle was dried with sterile paper so it could adhere to the flask more easily. One flask was filled with 4-10 tissue pieces with a space between them to allow the cells to migrate. The flasks with the tissues without any media were placed in the incubator in order to allow the tissues to stick onto the surface of the flasks for about 2-3 hours (Figure 2-2). After incubation, Dulbecco's modified Eagle's medium containing 10% FBS, 1%

penicillin/streptomycin and 1% L-glutamine was used as the supplemented media and this was placed into the flasks and kept in the incubator until confluency was reached. The media in flasks was changed every other day. All cell culture flasks were kept in an incubator at a constant 37° C, 5% CO2 and 95% humidity.

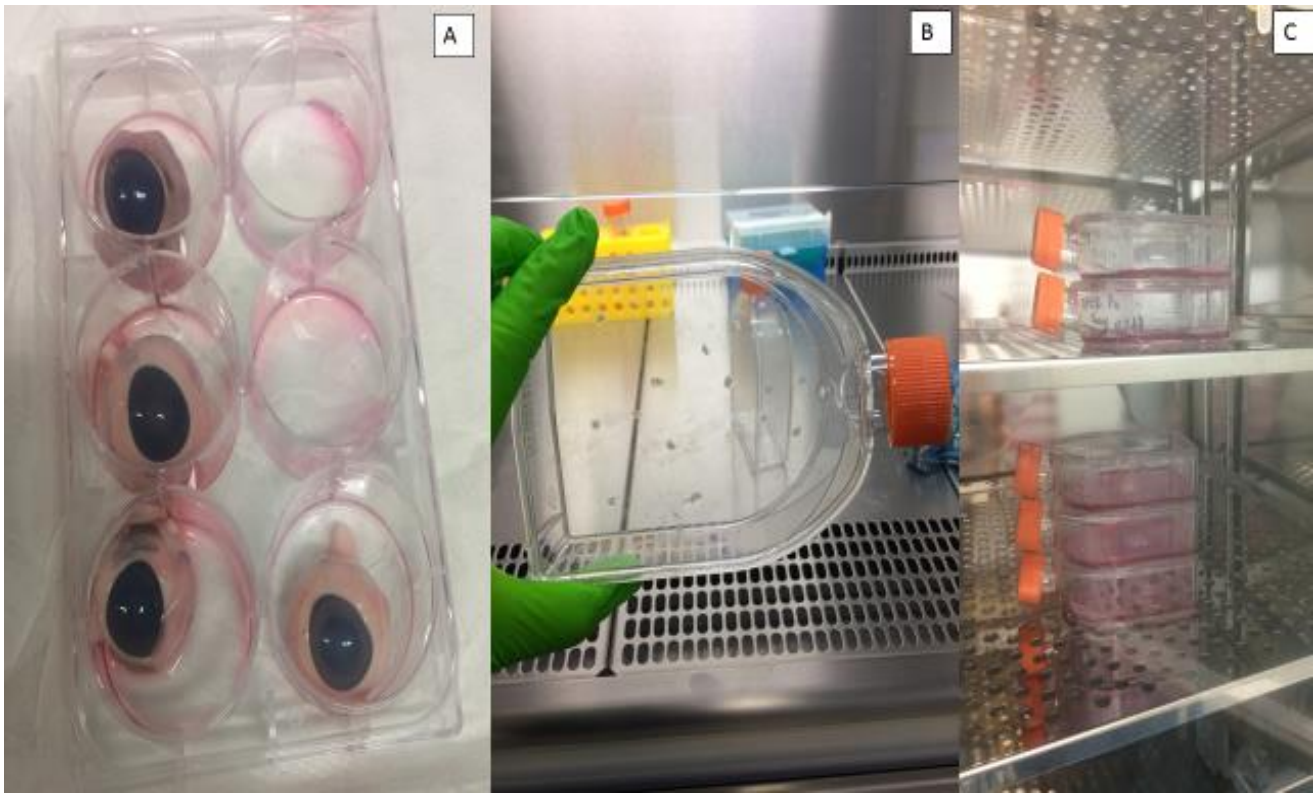

Figure 2-1.A. Porcine eyes are shown ready for dissection, just after the removal of excess fat and blood around the ocular surface. B. The conjunctival tissue in the tissue culture flask after being washed with 3% antibiotics. The conjunctival tissue was left for 2 hours in the incubator until the tissue stuck to the bottom of the tissue culture plate. C. After two hours, the flasks were topped with media containing DMEM, 10% FBS, 1% Antibiotics, 1% L-glutamine.

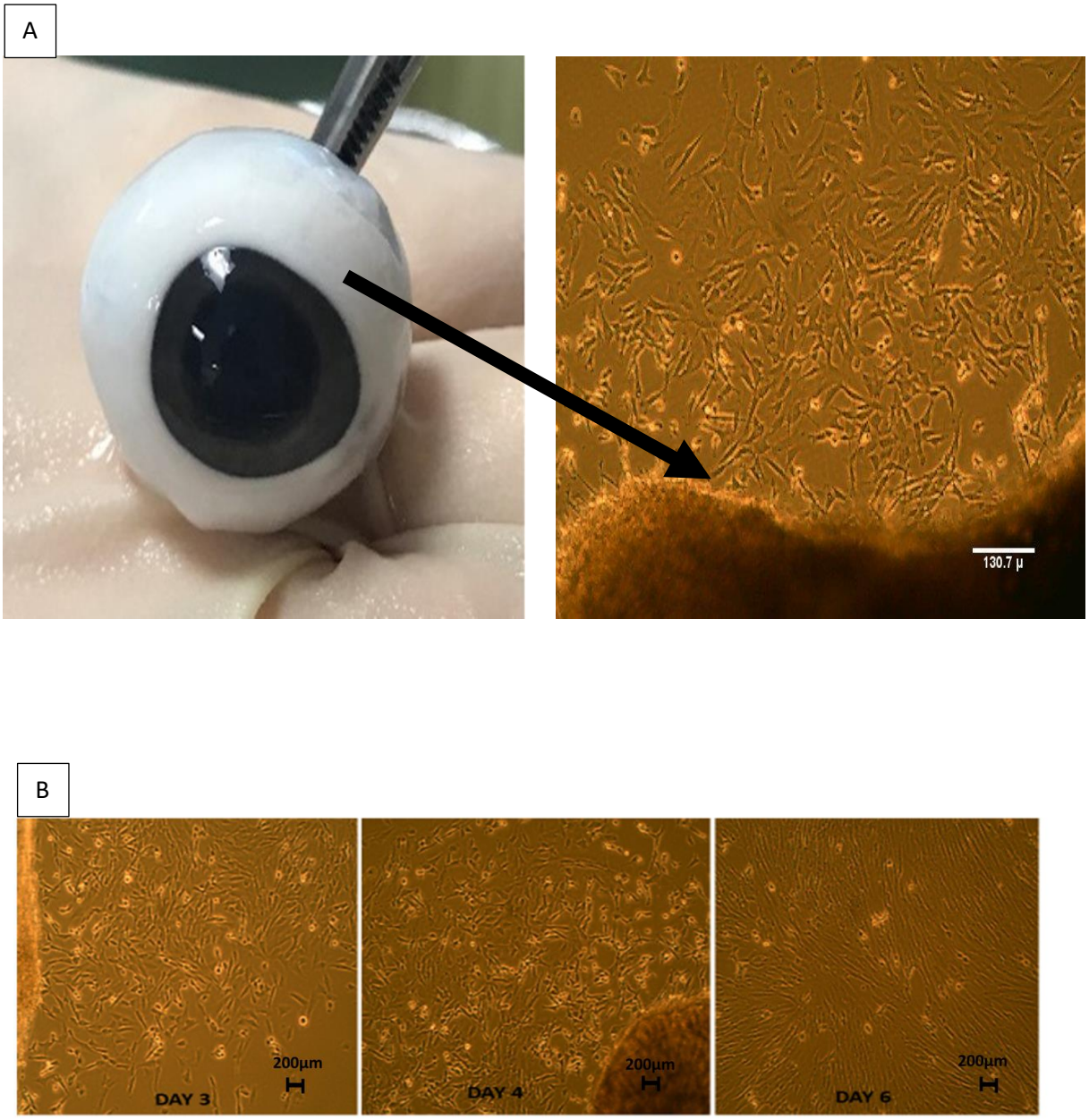

Figure 2-2. A. The porcine eye and the migration from the conjunctiva tissues. The tissues were taken from the conjunctiva of the eye and placed into the tissue culture flasks. The fibroblasts were migrated from the explant seen in the right-hand side image. The arrow indicates the place where the explant was taken. B. Migration of the fibroblasts from the explant was observed gradually with different time points from day 3 to day 6.

2.2 Cell Culture

All cell culture procedures were performed using T25 or T75. A seeding density of 5 x 10^3 cells/cm^2 for all the cell culture flasks, petri dishes and well plates was kept constant throughout all the experiments

presented in the thesis. Once confluency was reached in the flasks, passaging took place with enzymatic cleavage using trypsin enzyme. Details can be found in the following section.

2.3 Cell Count

Cell counts were carried out when the culture flasks had 80% confluency. The cell count started by discarding the media and washing the flasks with PBS. 0.25% Trypsin-EDTA (Ethylenediaminetetraacetic acid) (Lonza, Cambridge, UK) was placed into the flasks (3ml for T25 AND 8ml for T75), and the flasks were incubated for 4 to 8 minutes in the incubator at 37 °C at 5 % CO_2 until all the cells were lifted off from the flasks. A greater volume of cell culture medium than trypsin enzyme was added to ensure inactivation of the trypsin enzyme. The trypsin-media solution was collected and centrifuged for about 5 minutes at 300 x g to form a cell pellet. The pellet was resuspended in the supplemented medium (1 to 10ml) and 200 µl of the solution was taken to be counted with the Nucleocounter. Via-1 Cassettes is coated with Acridine Orange and Dapi staining to detect live and non-viable cells respectively.

2.4 NaCl-Induced Hyperosmolar Stress

Hyperosmolar stress was created using additional NaCl added to the already supplemented medium as described above. The different hyperosmolar medium was prepared to mimic the severity of dry eye condition; 30Mm, 50Mm, 70Mm and 90Mm were added from 1M of NaCl to the supplemented medium after the cells were cultured in culture plates described by Li et al., stated [215]. The osmolarity was measured by using Gonotec Osmomat 3000 freezing-point osmometer (Gonotec GmbH, Germany). Each formulation was tested in triplicate, measuring the vessels filled with 50 µL of the given formulation. The probe was rinsed with dH_2O and dried between the samples.

The media of the cells were changed every three days. The cells were cultured in hyperosmolar medium 48 hours prior to any other treatment.

2.5 Wound Healing Assay

Scratches were created with different pipette tips depending on the well plate due to the area differences in the well plates. For 96 well plates, a 200 µl pipette tip was used while for the petri dishes and 24 well plates the 1 ml pipette tip was used. A vertical scratch was made from the top to the bottom of each plate or petri dish [230]. Just before light irradiation, the scratch assay was performed both in cells cultured within the normal supplemented medium and hyperosmolar medium. Post-scratch, the cells were washed with PBS to get rid of any debris created during the scratch.

The cells were visualised by either using light microscopy (EVOS, Life Technology, Loughborough, UK) or fluorescence microscopy (Leica, London, UK) with the live-dead assay. From the images that were taken at different time points to monitor wound healing, the wound area was calculated using Image J by the same examiner. The images named without details of the condition until they are analysed. At least three different images for each condition were counted and the average of the area was taken.

2.6 Live/Dead Assay

The viability of the conjunctival fibroblasts was visualised by using a Live/Dead viability kit (Invitrogen, Loughborough, UK) which labels the dead cells as red and the live cells as green. Live cells were stained with the green fluorescent Calcein-AM due to their common feature of ubiquitous intracellular esterase activity and unimpaired membrane. Red fluorescent ethidium homodimer-1 stains the destructed plasma membrane as a common indicator of cells that have been under cytotoxic conditions.

The concentration of the dye was kept constant independent from the volume of the dye solution required in the different cell culture plates. 20 µl of ethidium bromide and 5 µl of calcein-AM were used in 10 ml of PBS. The cell culture medium from the well was discarded followed by washing with PBS. Live/Dead assay was placed into the wells depending on the preferred well plate. Following the manufacturer's protocol, the cells were incubated with the recommended concentrations of calcein AM and ethidium homodimer (EthD-1) for 30 minutes in the dark at room temperature. Post-incubation, the cells were washed with PBS and then imaged on a fluorescence microscope (Leica, London, UK).

2.7 MTT Assay

3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT; Sigma-Aldrich, UK) is a colourimetric assay which is an accurate indicator of cellular viability. MTT is a yellow solution which is converted to water-insoluble dark blue MTT formazan by dehydrogenase enzymes in the mitochondria of living cells. The formazan crystal's absorbance is directly proportional to the number of metabolically active cells. The MTT assay was used according to the manufacturer's instructions. Briefly, the cells were incubated with 0.5 mg/ml MTT for two hours after removal of their medium. The MTT solution was then aspirated and the cells were washed with PBS. 100 µl dimethyl sulfoxide (DMSO; Sigma-Aldrich, UK) was added to each well of 96 well plates for one hour and kept in an incubator. The plate was then read on a plate reader (Multiskan GO, Thermo Scientific, UK) measuring the absorbance with a wavelength of 570 nm. The absorbance values were normalised according to the control absorbance values as 100 %. The mean average of the control values was calculated. The equations were applied as follows:

$$= \frac{\text{Actual Absorbance}}{\text{Average Control Absorbance}} * 100$$

Equation 2-1. Absorbance values were normalised using this equation shown. The percentage of the difference in cell viability was calculated after calculation of the relative percentage of each condition.

This equation gives the relative percentage of cell viability according to the control (untreated) samples. Each condition had at least six repetitions for each well plate.

2.8 Presto Blue Assay

PrestoBlue assay was used for the same purpose as the MTT assay described previously. Viability differences for earlier experiments were calculated using the MTT assay however, thereafter PrestoBlue was used due to being more stable and faster than the MTT assay. PrestoBlue assay provides a quantitative indication of the cell viability by reducing the environment of the live cells and giving a colour change from purple to red.

The medium of the cells was discarded and the cells were washed with PBS. The PrestoBlue concentration was diluted from 10X to 1X with the culture medium used for the cells as manufacturer protocol recommended. For each well of the 96 well plates, 100 µl presto blue solution was used. The plates were cultured 2 hours in the incubator at 37°C at 5 % CO_2. The change of the colour was measured the by fluorescence plate reader (Spectra Max Gemini EM, UK) at 535-560⁄590-615 nm as excitation and emission respectively. The fluorescence values were normalised according to the control values as 100 %.

2.9 Statistical Analysis

At least three samples were used for each variable. Raw data were entered into the spreadsheet and sorted initially in Microsoft Excel. When it is not applicable on Excel, data was transferred to SPSS software. The normality was checked with the skewness and kurtosis test to determine the suitable test. Student t-test, One-way or two-way ANOVA was used throughout the thesis. Throughout the thesis mean and standard deviation values for each variable are provided in the bar graphs. In graphs, standard deviations are represented by error bars. A p-value below 0.05 was taken as being statistically significant.

CHAPTER 3: LIGHT EXPOSURE ON CONJUNCTIVAL CELLS UNDER HYPEROSMOLAR STRESS

3 LIGHT EXPOSURE ON CONJUNCTIVAL CELLS UNDER HYPEROSMOLAR STRESS

Overview

Tear hyperosmolarity is one of the important core mechanisms in dry eye and is caused by an abnormal increase in evaporation from the ocular surface [53]. The schematic vicious cycle of dry eye (Figure 1-2) indicates how inflammation contributes to and worsens the severity of dry eye [1]. It is not only an increase in evaporation that could lead to hyperosmolarity but also any other condition in the vicious cycle. Hyperosmolarity is a common pathology of both evaporative dry eye and aqueous-deficient dry eye in which cataract surgery initiates or worsens the dry eye condition [60]. There have been only a few studies in the scientific literature that have investigated how light from an operating microscope during cataract may have an effect on the ocular surface. This chapter investigates the effect of light irradiation from a laboratory microscope initially as a pilot study followed by an operating microscope in simulated dry eye disease in vitro.

Additionally, scratch assays were performed to evaluate the effect of light radiation on wound healing. The wound healing rate was analyzed with different techniques at different time points. Assessments of live-dead staining, cell viability, apoptosis rate, inflammation rate and cell diameter were carried out to gain a better understanding of the effects of ophthalmic surgery on ocular surface cells.

3.1 Introduction

Cataract surgery is one of the risk factors for dry eye disease [130,147]. The significance of the incidence is well established from various studies. The reason for its occurrence or why it worsens in patients with pre-existing dry eye is still not fully understood. As a surgical procedure which is performed on millions of people worldwide each year, the need for understanding patient dissatisfaction post- surgery is crucial.

Light exposure from the operating microscope may be one of the factors leading to dry eye disease after cataract surgery. The correlation between duration of the cataract surgery and ocular surface damage in terms of goblet cell density reduction was found (r^2= 0.65) [117] even though the surgery typically takes around 10 to 15 minutes nowadays [105]. Further to this, during cataract surgery, normal blinking is also prevented which may potentially intensify the ocular surface damage by the exposure of light directly to cornea and conjunctiva even though it is rinsed during the surgery. Light has been known to cause phototoxic effects on the retina, through photothermal [254], photomechanical [255] and photochemical [256] mechanisms, all of which have been studied extensively and are well documented in the published literature [254,257–261]. However, until now, there have only been a few studies investigating the effect of operating microscope exposure on the ocular surface tissues in vitro and in vivo [116,153,171] even though cornea, conjunctiva and tear film are the first structures that the surgical microscope light encounters.

The effect of the operating light microscope on the rabbit's ocular surface was observed by Hwang et al. [116]. Their analysis of the ocular surface after light exposure showed decreased tear production, devitalized conjunctival and corneal cells, reduced goblet cell density, and an increase in IL-1β as one of the markers of inflammation. Although the rabbit eyes demonstrated a more aggressive inflammatory response compared to the human eye, these findings suggest that light might be a significant factor to consider in the possible aetiology of post-operative dry eye [262]. The pathogenesis of dry eye and its association with light irradiation is still not clear since limited emphasis has been given to light irradiation and ocular surface impact.

Dry eye pathogenesis itself has been studied extensively, however being a multifactorial disease, it is still not fully understood. Various in vitro models have been used to investigate a number of pathological mechanisms thought to play a role in dry eye. Hyperosmolar stress and inflammation have been stated as core mechanisms of dry eye, therefore, most of the in vitro studies are based on those two stressors [206,213,263]. Most of the previous in vitro studies regarding dry eye have used predominantly corneal epithelial cells and some have used conjunctival epithelial cells for experimentation. Conjunctival cells were found to be affected more adversely by blue light irradiation than the corneal cells under the same hyperosmolar stress [168]. This might be due to the cornea's immune privileged and avascular nature compared to the conjunctiva's highly interconnected immunocompetent cells in vivo that result in conjunctival cells being more responsive to the light [168]. Moreover, the conjunctival response carries significance for dry eye disease in terms of ocular immunology, as the conjunctiva is the first location of ocular surface inflammation and naturally contributes more to the inflammation response than the cornea [168,263]. Therefore, the conjunctiva has been found to be more responsive than the cornea to light [168].

Conjunctival epithelial cells damaged in the dry eye disease may expose underlying conjunctival fibroblasts to the external environment including hyperosmolar tear stress. Interestingly, no study has explored conjunctival fibroblast' behaviour in this regard. Conjunctival fibroblasts have been found to be a crucial cell population in the conjunctival inflammation response [216,264] and further investigation of their role in dry eye is warranted. Thus far, little attention has been paid to the role of those cells in dry eye disease.

This study aims to elucidate the conjunctival fibroblasts response in vitro to potentially harmful light exposure from an operating microscope. Hyperosmolar stress was used in vitro to simulate pre-operative and postoperative dry eye. The influence of hyperosmolarity was investigated over a range of concentrations to reflect the variations over the ocular surface noted in the academic literature [168,213,226]. In addition, to simulate hyperosmolar stress, scratch assays were performed to assess wound healing with light exposure mimicking the damage to the conjunctiva during ocular surgery due

to the use of a speculum and/or antiseptics. Conjunctival fibroblasts were analysed by their cell viability, morphology, cell size, apoptosis rate and inflammatory cytokine level of IL-6.

3.2 Materials and Methods

3.2.1 Cell Culture

Porcine conjunctival fibroblasts were used from p2 to p4 for this study. Detailed methodology for the isolation of conjunctival fibroblasts from the explant tissue can be found in Chapter 2 Explant Dissection and 2.2 Cell Culture. A black 96 well plate with the clear bottom (Sigma-Aldrich, Dorset, UK) and petri dishes (10 mm) (Costar, Nottingham, UK) were used for the experiments with a seeding density of 5x 10^3 cells/cm^2. Cells were cultured in DMEM (Gibco, Cambridge, UK) supplemented with 10% FBS (Sigma-Aldrich, Dorset, UK) and 1% antibiotics (Lonza, Cambridge, UK) until they reached more than 70% confluency, which usually took 96 hours.

3.2.2 Cell Characteristics

The growth rate was calculated according to the total viable cell count from each passage number as opposed to the initial seeding number. The population doubling rate was calculated according to growth rate obtained by using the following equation:

n= (log Y- log X) * 3.32,

Equation 2. Population doublings equation. In the equation Y refers to total viable counts and X refers to the initial seeding number.

where Y is the total viable cell count and x is the initial cell number which were put into the flask [37]. Cell counts were taken from the T75 flasks (SLS, Nottingham, UK) with different passage number at 10 days of culture time after seeding.

3.2.3 Actin Staining

Fibroblasts were stained with phalloidin (Invitrogen, Cambridge, UK) to detect the actin cytoskeleton of the conjunctiva fibroblasts. The cells were fixed with the 4% paraformaldehyde (Sigma-Aldrich,

Dorset, UK) in 1x PBS (Sigma-Aldrich, Dorset, UK) for 15-20 minutes at room temperature. Cells were then washed twice with staining buffer. Permeabilisation of the cells was carried out with Perm/Wash buffer for 5 minutes at room temperature, followed by repeating the washing step with staining buffer. Blocking buffer was applied (3 droplets/well) for 45 minutes in the dark at room temperature. After the incubation at room temperature, the blocking buffer was removed. Phalloidin working solution (Phalloidin Stock solution – 10 nmol in 0.5 mL D-PBS; Working solution: 20 – 30 µL of stock solution in 1 mL of staining buffer) was applied and left for incubation about 45 minutes at room temperature in the dark (200 µL/well). The phalloidin solution was removed and the wells were rinsed once with PBS and once with deionized water. Aluminium foil was used to cover the plate at all times to avoid fluorescence bleaching. Fluorescence images were visualized with a fluorescence microscope (Leica, London, UK).

3.2.4 NaCl-induced hyperosmolarity

The NaCl-induced hyperosmolar medium was prepared with the addition of 30mM, 50mM, 70mM or 90mM (Sigma-Aldrich, Dorset, UK) to the supplemented medium (DMEM low glucose 10 % FBS, 1 % Penicillin and Streptomycin). Osmolarity was measured with an Osmometer (Osmomat 3000, Gonotec, Berlin, Germany). Each formulation was measured three times. Comprehensive details can be found in Chapter 2, 2.4 NaCl-Induced Hyperosmolar Stress.

3.2.5 Scratch assay

A vertical scratch from the top to bottom of each well was created using a sterile 200 µl pipette tip in the 96 well plates and a 1000 µl pipette tip for the Petri dishes. The wells were washed with PBS to remove the floating debris.

3.2.6 Light exposure and light intensity measurements

Two types of the microscope were used; an AE200 light microscope (Motic, Wetzlar, Germany) and an ophthalmic microscope OPMI 1 FR pro operating microscope (Zeiss, Germany). The maximum intensity of the light from both microscopes was used and this was kept constant throughout the experiment.

The distance between the sample and the light source was also kept constant. The distance from the sample to the laboratory light microscope was 7.3 cm which were the stage of the microscope was located. The distance from the sample to the operating microscope was arranged as 15 cm which was measured when the surgeon was operating the cataract on ex vivo porcine model.

The spectra of the light sources were measured with a USB 2000+ spectrometer (Ocean Optics, Ostfildern, Germany) to measure between 350 and 1000 nm and the NIRQuest 512 spectrometer (Ocean Optics, Ostfildern, Germany) to measure between 900 and 1700 nm to observe infrared light. The spectrometers were calibrated against either deuterium or tungsten-halogen standard lamps that had been calibrated by the Physikalisch-Technische Bundesanstalt (PTB), the national metrology institute of the Federal Republic of Germany.

3.2.7 Experimental Set up

3.2.7.1 *Preoperative Dry Eye Model*

Experimental design of the pre-operative dry eye is shown schematically in Figure 3-1. Hyperosmolar medium (370, 412 and 480 mOsm/kg) and control (328 mOsm/kg) medium were renewed before 48 hours of the scratch assay. Scratch assay was performed and 10 minutes of light exposure was carried out with the light source immediately after the creation of the scratch. The medium of the wells was renewed with the same culture medium until the termination.

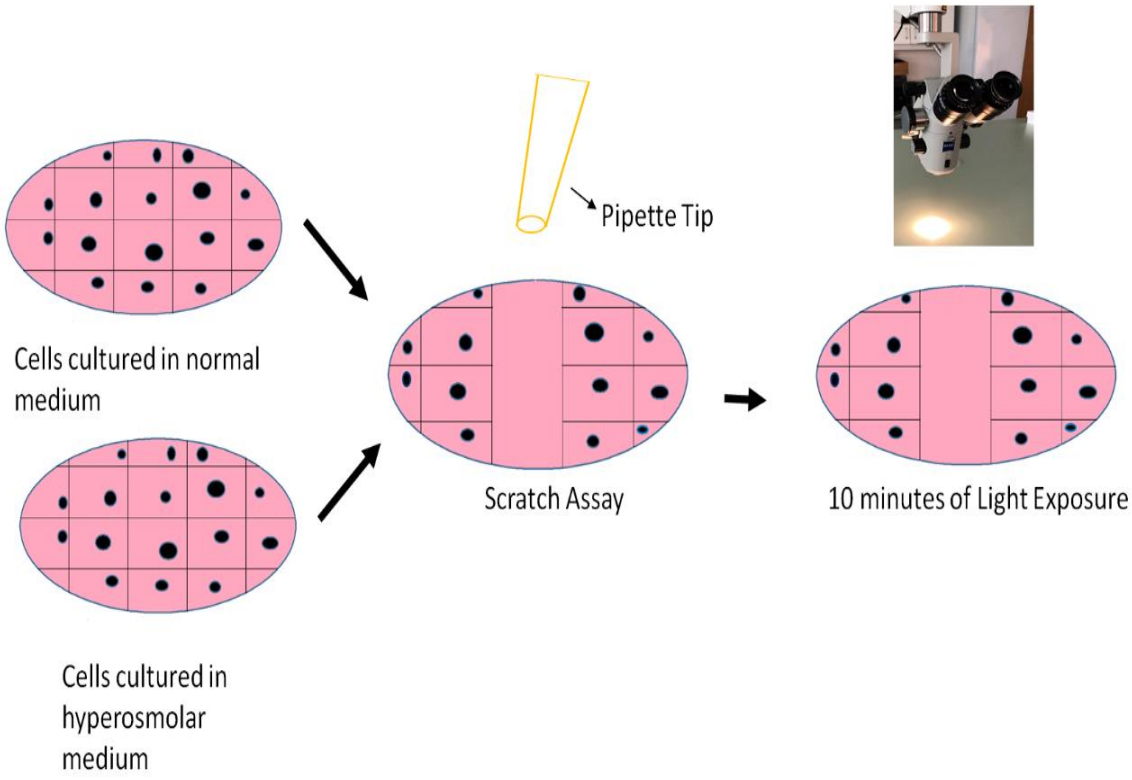

Figure 3-1. Schematic presentation of the experimental design to mimic pre-operative dry eye before light exposure. The cells were cultured in a hyperosmolar medium or control medium for 48 hours before the scratch assay.

3.2.7.2 *Postoperative Dry Eye Model*

A schematic impression of the post-operative dry eye experimental model is shown in Figure 3-2. The cells were cultured in control medium until the scratch assay. Scratch assay was followed with 10 minutes of light exposure. The medium was changed after the light exposure to either hyperosmolar or control medium.

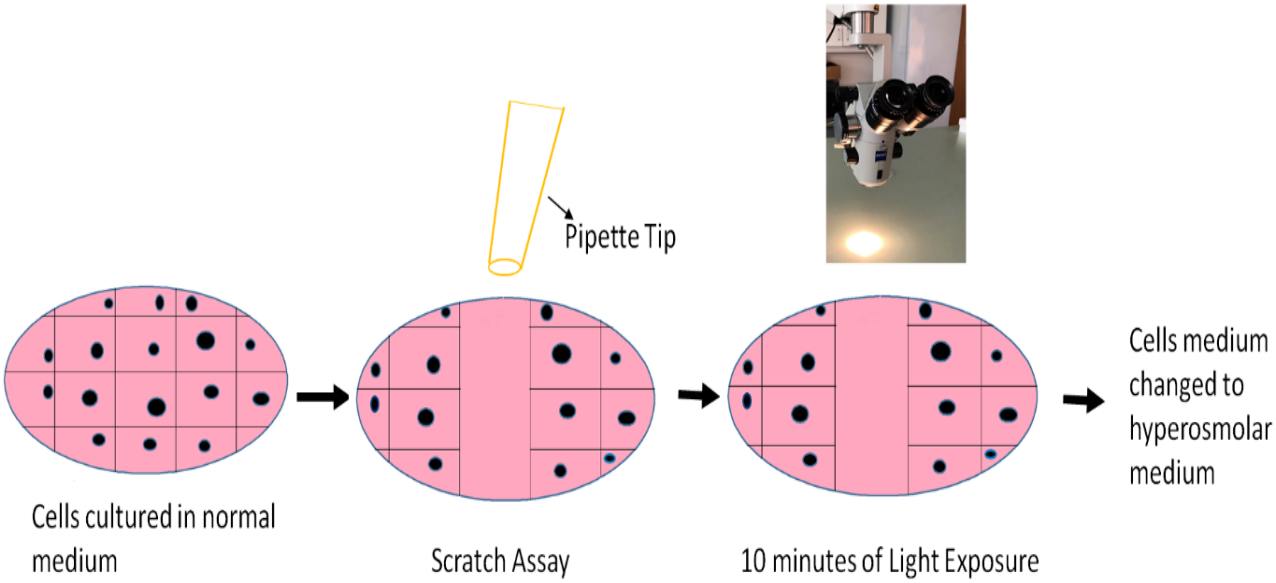

Figure 3-2. Experimental design of a post-operative in vitro dry eye model. After the scratch assay was performed, 10 minutes of light exposure was carried out. The media were changed to hyperosmolar medium thereafter.

The light intensity of the laboratory light microscope was measured at 10 000 lux (TV335, Testboy, Germany) while the exposure was carried out.

3.2.8 Wound Closure Rate Calculation

The wound area was measured using Image J (Version 1.51k National Institutes of Health, Bethesda, MD, USA) from each time point after irradiation with the light microscope. The area of the wound was calculated for each image. The average of the three different pictures for each time point and condition was calculated.

3.2.9 Viability Assays

3.2.9.1 *Live/Dead Viability Kit*

The viability of the conjunctival fibroblasts was visualised using a Live/Dead viability kit (Invitrogen, Cambridge, UK) which labels the dead cells red and the live cells green. The cells were washed with PBS when they were ready to be terminated for analysis. Live/Dead assay solution was prepared according to the manufacturer's protocol with Calcein Am and Ethidium Homodimer. Cells were

washed with PBS before incubation with the assay solution. The wells were incubated at 37 °C and 5% CO_2 in the dark for 30 minutes. They were then washed with PBS. The images were visualised by using a fluorescence microscope (Leica, London, UK). Further details can be found in Chapter 2, 2.6 Live/Dead Assay.

3.2.9.2 *MTT Assay*

For the quantitative analysis, MTT (Sigma-Aldrich, Dorset, UK) and PrestoBlue (Invitrogen, Cambridge, UK) were used to observe differences in cell viability in the cells. Both assays were used according to the manufacturer's instructions. For the MTT assay, briefly, the fibroblasts were incubated with 0.5 mg/ml MTT for two hours. The MTT solution was then aspirated. Each well was covered with 100 µl of DMSO (Sigma-Aldrich, Dorset, UK) for one hour. The plate was then read on a plate reader (Multiskan Go, Thermo Scientific, Loughborough, UK) measuring absorbance with a wavelength of 570 nm.

MTT standardization was carried out with different cell density values in order to ensure that cell seeding density could be measured within the MTT range. This avoided over-saturation and not having enough cells to be read (Figure 3-3). Cell density was chosen as $5x\ 10^3$ cells for the MTT assay according to the standardization of the MTT assay. All MTT results were analysed with the plate reader at 570nm. All absorbance values were normalised according to the control average absorbance and are shown as the relative percentage throughout this chapter.

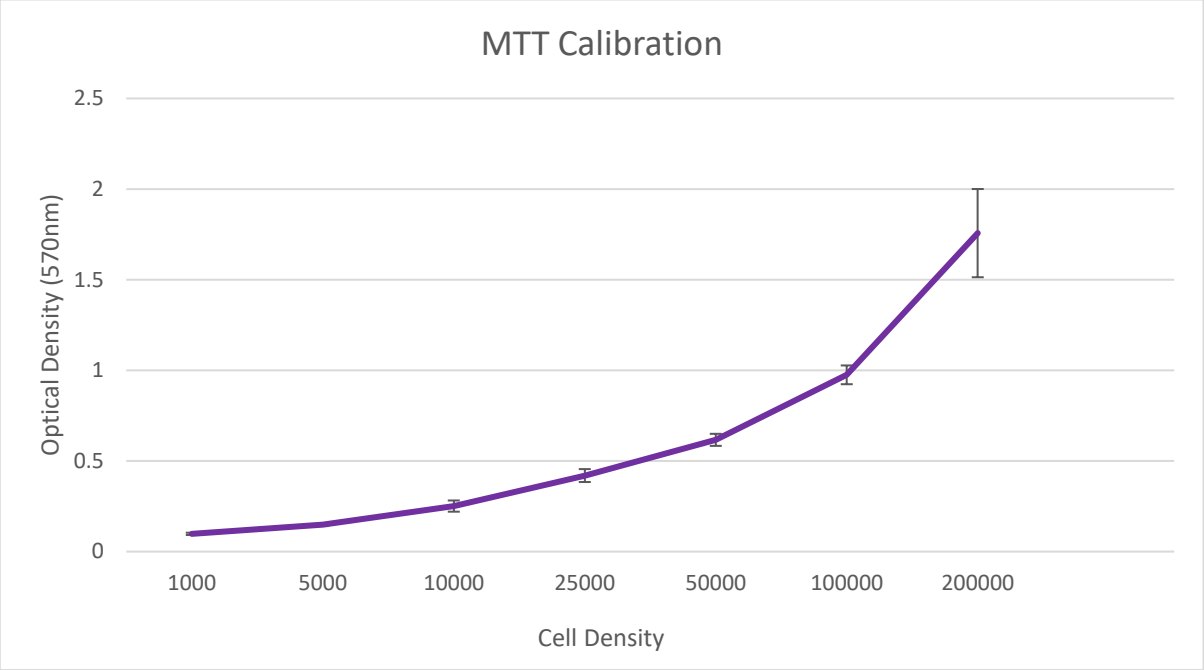

Figure 3-3. The MTT standardization with different cell density on 96 well plates. 5 x10³ cells/well were chosen for the MTT analysis for the rest of the MTT results.

3.2.9.3 *PrestoBlue Assay*

PrestoBlue was also used to detect viability. This has the advantage of not requiring the termination of the culture. PrestoBlue (10X) solution was diluted with the supplemented medium in order to make the 1X presto blue solution. The cells were rinsed with PBS and incubated for 2 hours with the PrestoBlue solution in the dark at 37 °C and 5% CO_2. Fluorescence was measured with an excitation wavelength of 560 nm and an emission wavelength of 590 nm on a plate reader (Spectra Max Gemini EM, UK).

Quantitative cell viability was measured with PrestoBlue assay due to the availability of the MTT solution. Calibration of the assay was carried out with different seeding cell numbers shown in Figure 3-4. The cell density was also chosen as 5 x 10^3 for the presto blue assay.

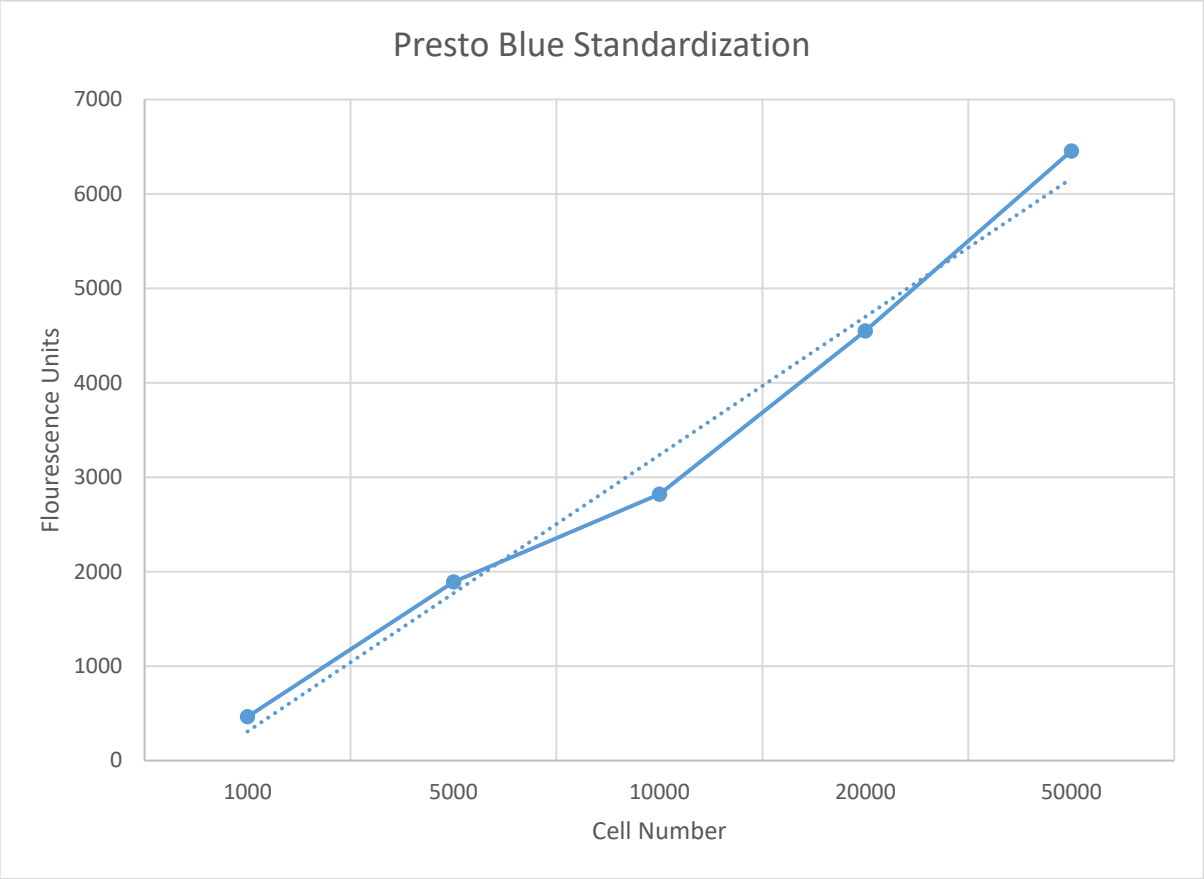

Figure 3-4. PrestoBlue calibration curve is shown with a different cell number for 2 hours of culture. Seeding density number of 5 x 10^3 was chosen as the preferred density.

3.2.10 Cell Size Quantification

The conjunctival fibroblasts were seeded into the 10 mm Petri dishes (Thermo Fisher, Loughborough, UK) and terminated at the 24-hour time point after irrigation. The media was aspirated and cells were washed with PBS. Enzymatic cleavage to detach of the cells from petri dish was achieved with 1ml of trypsin and the cells placed in an incubator until all the cells were lifted off from the petri dish. Trypsin was inactivated with a supplemented medium, added at a higher volume than that of the trypsin in order to ensure its inactivation. The cell suspension solution was collected in an eppendorf and centrifuged at 300 x g for 5 minutes. The cell pellet was resuspended in 1 ml media and counted with the nucleocounter Via1-Cassettes (Chemometec, Lillerød, Denmark). Cell size, viability and the count of live and dead cells were given by the nucleocounter. Each condition was performed in triplicates.

3.2.11 Annexin V Assay

Petri dishes were used to analyse the annexin V levels. Once petri dishes reached 70% of confluency, their media were changed to hyperosmolar versions media following the preoperative dry eye model outlined in Figure 3-1 The petri dishes were terminated for analysis at the 24-hour time point after the light exposure. Enzymatic termination with trypsin was carried out to obtain the cell count. For each repetition, three petri dishes from the same condition were combined in order to reach the cell number required for the analysis by the nucleocounter annexin V assay. After the cell count, samples were centrifuged again at 300 g for 5 minutes. The supernatant was discarded and resuspended in 100 µl of Annexin V binding buffer (Chemometec, Denmark). The solution was left in the incubator at 37 °C for 15 minutes after the addition of 2 µl Annexin V-CF488A conjugate and 2 µl Hoechst 33342. Following incubation, the cell solution was centrifuged at 300 x g for 5 minutes. Resuspension was carried out with 300 µl Annexin V binding buffer after the supernatant removal. This step was repeated twice. After the supernatant was removed, 100 µl Annexin V binding buffer supplemented with 10 µg/ml Propidium Iodide (PI) (2 µl of PI to 100 µl binding buffer) was used to resuspend the pellet. Immediately after, 30 µl of each of the cell solution was loaded to NC-Slide A2™ (Chemometec, Denmark) to be read by the nucleocounter. The results of all the repetitions were analysed together in the plot manager taskbar and normalised according to the control values.

3.2.12 IL-6 Levels Detection

Porcine IL-6 Elisa kit (Sigma, UK) was used to analyse the IL-6 levels in the cell culture medium. Cell culture media were collected at the 24-hour time point, post operating microscope exposure. The culture media were centrifuged for 10 minutes at 400 x g. The supernatant was collected and stored at -80°C until the analysis was performed. The rest of the protocol was performed using the manufacturer's protocols (Sigma, UK).

Standards were prepared with serial dilution from 50 ng/ml to 40.96 pg/ml concentration with the 1X assay buffer (diluted with the deionized water from 5X to 1X). Each well was filled with 100 µl of the standards and samples in the 96 well plate and left for incubation for 2.5 hours at room temperature. The wells were washed 4 times with the 1x wash buffer provided (20x wash buffer was diluted with

deionized water to make the concentration 1x). For washing, 300 µl was placed into each well using a multichannel pipette. Complete removal of the wash buffer was provided with aspirating liquid then blotting it against a paper towel by inverting the plate. Each well was filled with 100 µl of 1x Biotinylated Detection antibody and incubated for 45 minutes at room temperature with gentle shaking. The washing step after the incubation was repeated four times as described in the previous step. Then the wells were filled with 100 µl of ELISA Colorimetric TMB reagent for 30 minutes in the dark with gentle shaking. Lastly, 50 µl Stop Solution was used to stop the reaction. The plates were immediately read by a plate reader (Multiskan GO, Thermo Scientific, Loughborough, UK) at 450 nm (adapted from the manufacturer's user guide).

3.2.13 Statistical Analysis

A minimum of three specimens for each variable was tested. The raw data was initially ordered in the Microsoft Office Excel software. The data were displayed as a mean value and the standard deviation of the mean. If necessary, data were transferred to SPSS for further statistical analysis. Normality was checked with the skewness and kurtosis test to determine the suitable test. Student t-test, one-way and two-way ANOVA (IBM SPSS v23, Chicago, IL, USA) were used.

3.3 Results

3.3.1 Confirming Fibroblasts Cell type and Cell Health

Porcine conjunctival fibroblasts stained positively for F-actin. Fibroblast morphology was observed as healthy, spindle-shaped, displayed in Figure 3-5.

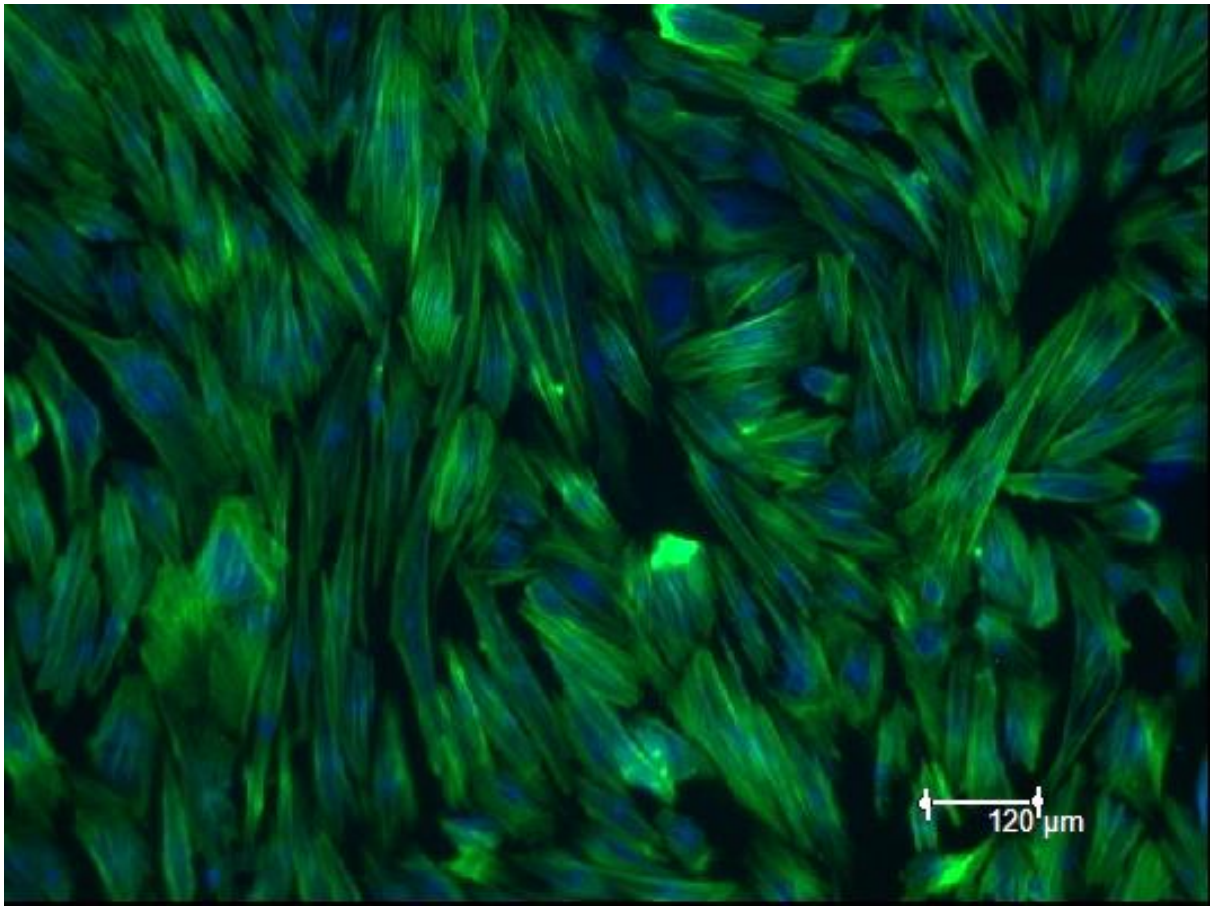

Figure 3-5. The fibroblast confirmation with actin staining. The fibroblasts stained with green due to phalloidin. The nucleus of the cells stained with Dapi as blue.

The population doublings rate is shown in Figure 3-6. Conjunctival fibroblasts from the explants were observed with different passage numbers and population doubling times. The slower proliferation rate was observed with increasing passage number.

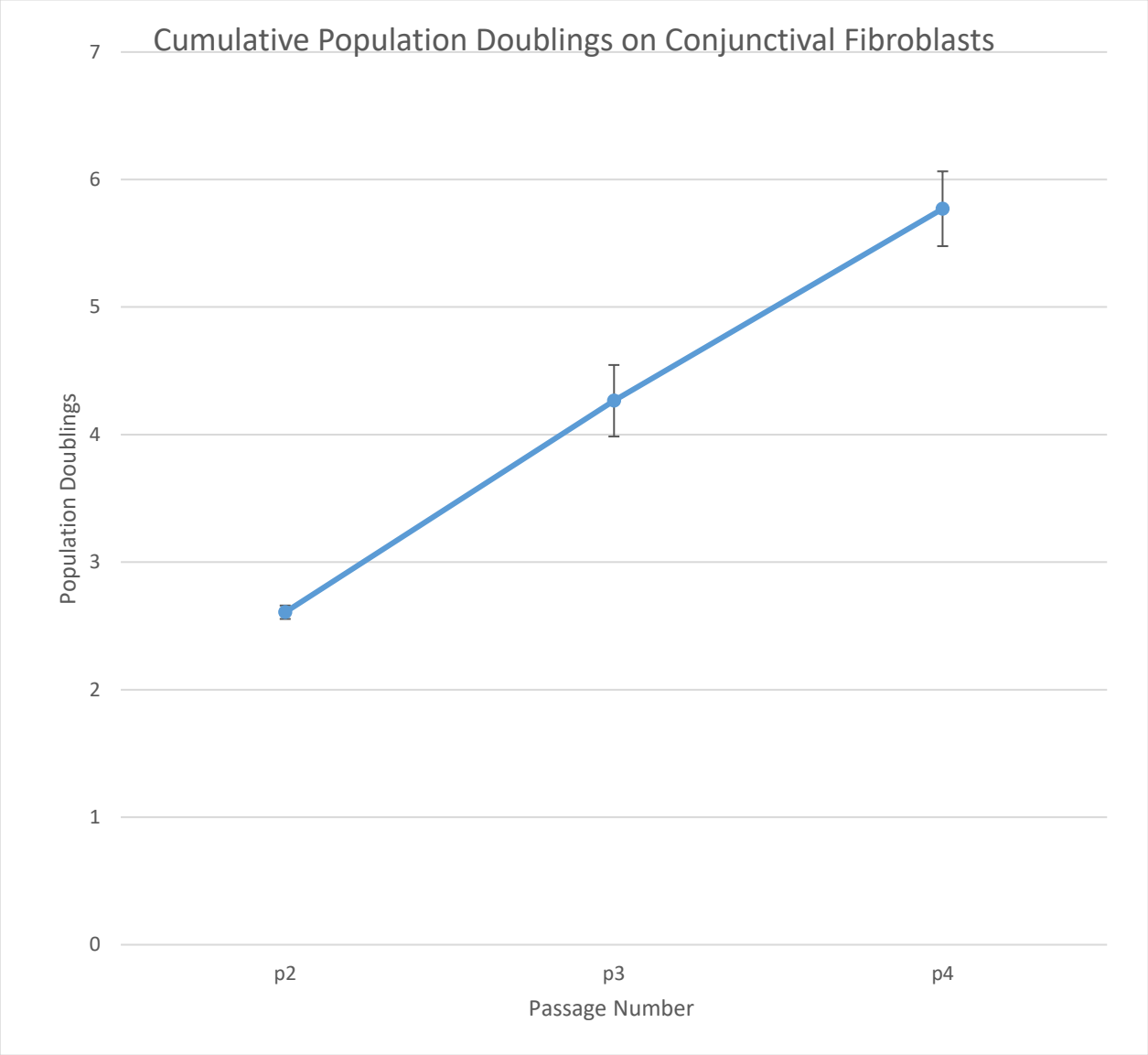

Figure 3-6. Conjunctival fibroblasts' population doubling rate with increasing passage number.

The results of light exposure were analysed in two sections as two different light sources were used; a laboratory and a clinical operating microscope. The first section refers to the results with the laboratory light microscope and the second part shows the results from the operating microscope.

3.3.2 Pilot Study

Laboratory microscope exposure of 10 minutes was tested throughout this section to approximate the duration of a typical cataract surgery procedure on conjunctival fibroblasts.

3.3.2.1 *Light Exposure and Light Intensity Measurements*

The light intensity of the laboratory light microscope (AE200, Motic, Wetzlar, Germany) was measured with a lux meter (TV335, Testboy, Vechta, Germany). The light microscope's light intensity was measured with a lux meter, as 10,000 ± 1000 lux. The spectra of the light microscope are indicated in Figure 3-7.

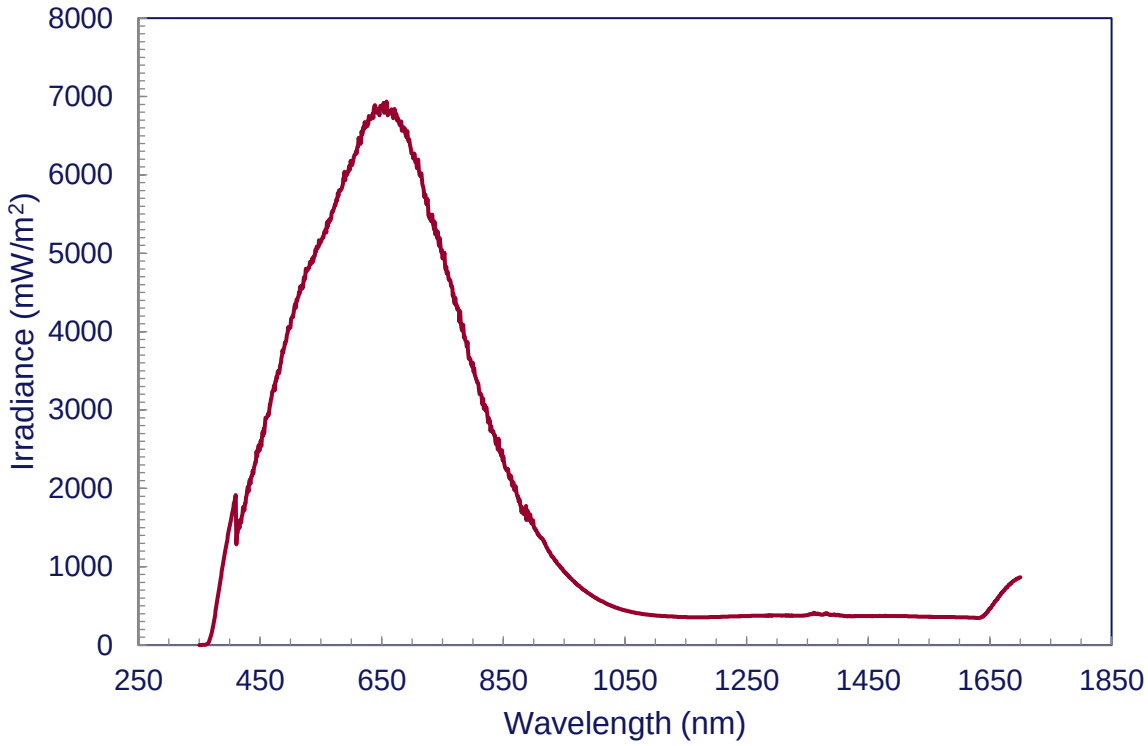

Figure 3-7. Spectra of the laboratory microscope.

3.3.2.2 *Wound Healing*

The scratch assay was created followed with 10 minutes of light irradiation from the laboratory microscope. The wound healing was assessed with images that belong to different time points: 1 hour, 3 hours, 6 hours, 12 hours, 24 hours and 48 hours, seen in Figure 3-8. Slower wound healing was observed when the cells were exposed to light. The most dramatic difference in wound area was achieved at the 12 and 24-hour time points. At the 48-hour time point, the wound area with both light-exposed and the dark cultures were almost closed.

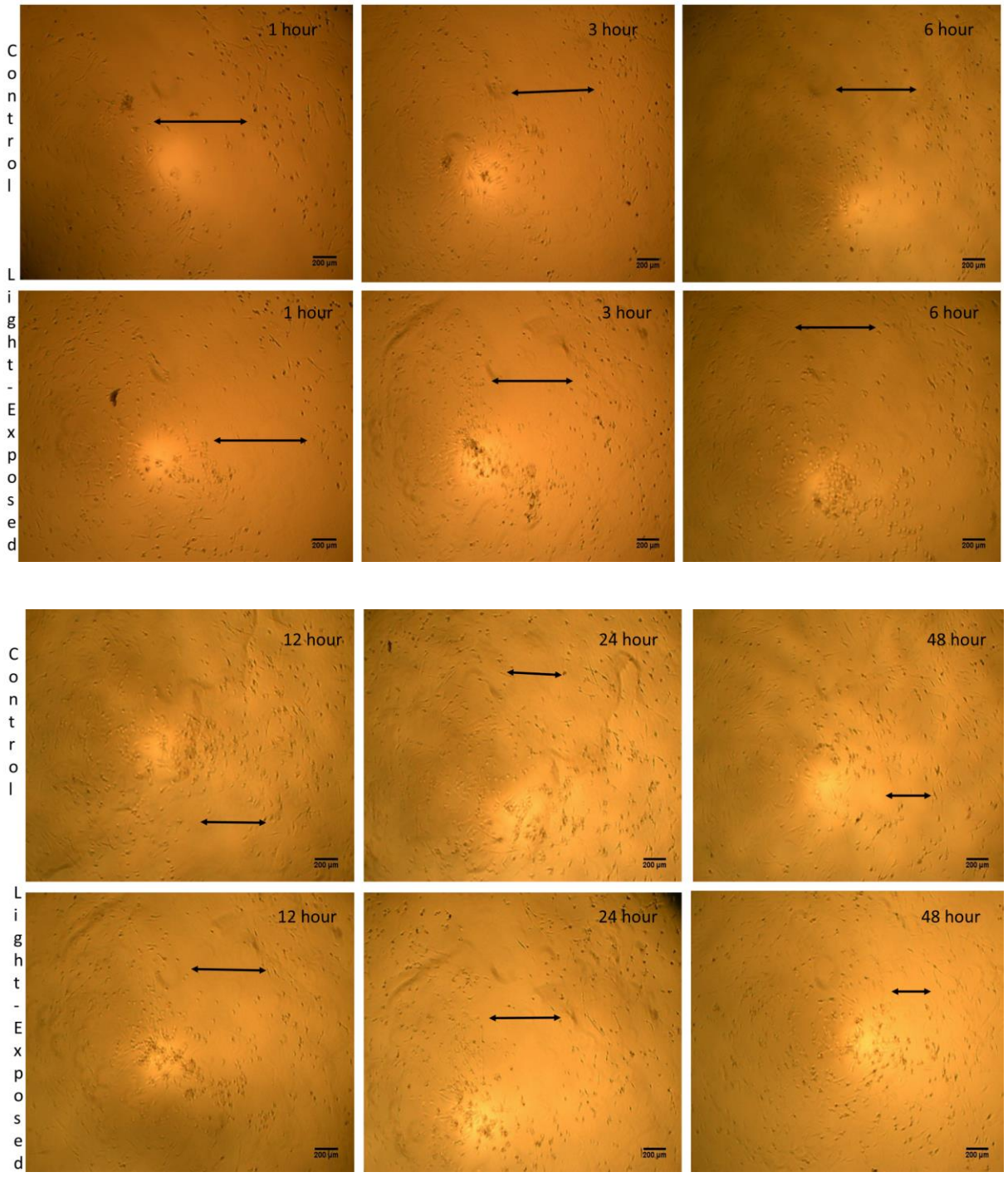

Figure 3-8 Light microscope images at time point 1, 3, 6, 12, 24 and 48 hours. The closure of the wound occurred gradually. All wounds were created with the same size pipette by the same investigator. All images were representative of more than 3 replicates. All the images were taken using the same microscope (Motic, Wetzlar, Germany)

The Live/Dead assay was used for visualisation of the wound healing with time, shown in Figure 3-9. The closure rate was similar in the light-exposed and control samples before the 24-hour time point (Figure 3-9). However, the closure rate started to become different after the 24-hour time point. The

light-exposed cells demonstrated a slower wound closure compared to the control condition at the 24-hour and 48-hour time point.

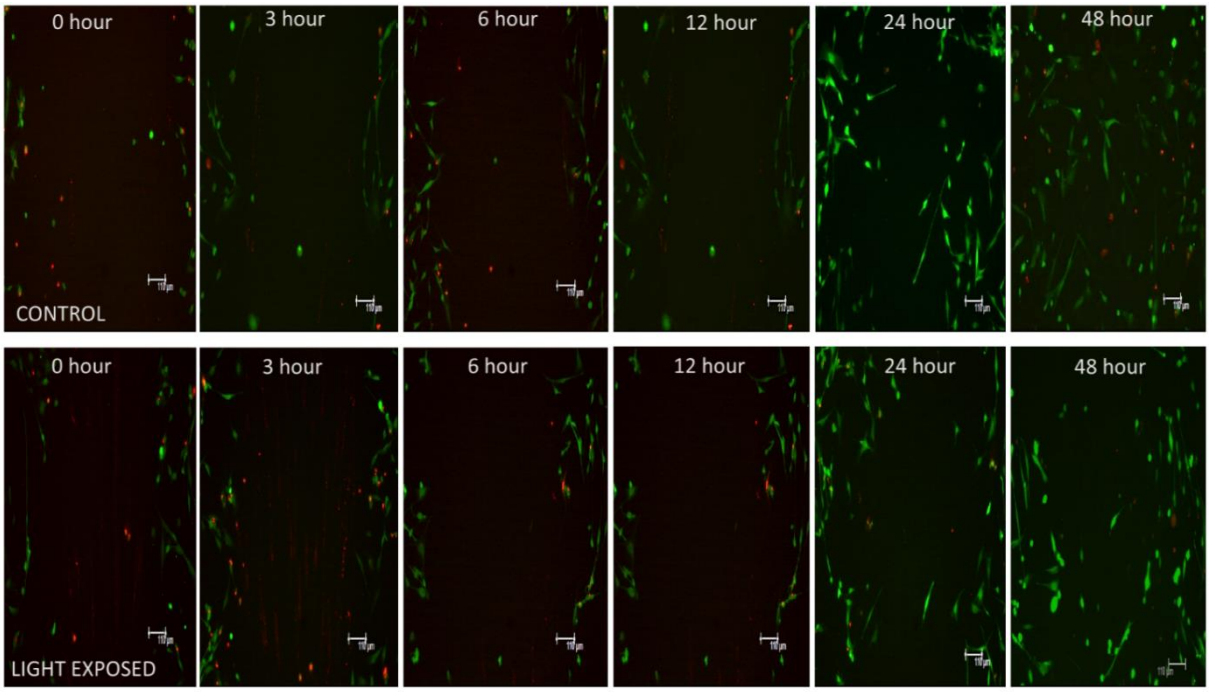

Figure 3-9. Live/Dead Assay with different culture time. Live-Dead staining images shown at different time points after the scratch was made. Image labelled as control represents the row of control images at different time points as 0, 3, 6, 12, 24 and 48 hours. Image labelled as light exposed represents the row of the light-exposed wound closure of cells at 0, 3, 6, 12, 24 and 48 hours.

Images of wound closure were used to calculate the area of the wound using ImageJ (at 0 hours, 3 hours, 6 hours, 12 hours, 24 hours after the scratch, in light-exposed cells and the cells without any light exposure (Figure 3-10). A significant difference between light-exposed and control cells was found at the 24-hour time point ($p = 0.02$). When applying the conservative Bonferroni correction, the difference did not remain statistically significant. The wound area for both conditions (light-exposed and control) at the 48-time point was not able to be assessed since the area of the wound was mostly closed at that time point. Before the 24-hour time point, the images did not show a significant difference in terms of wound closure which can be also seen with the live-dead assay in Figure 3-9.

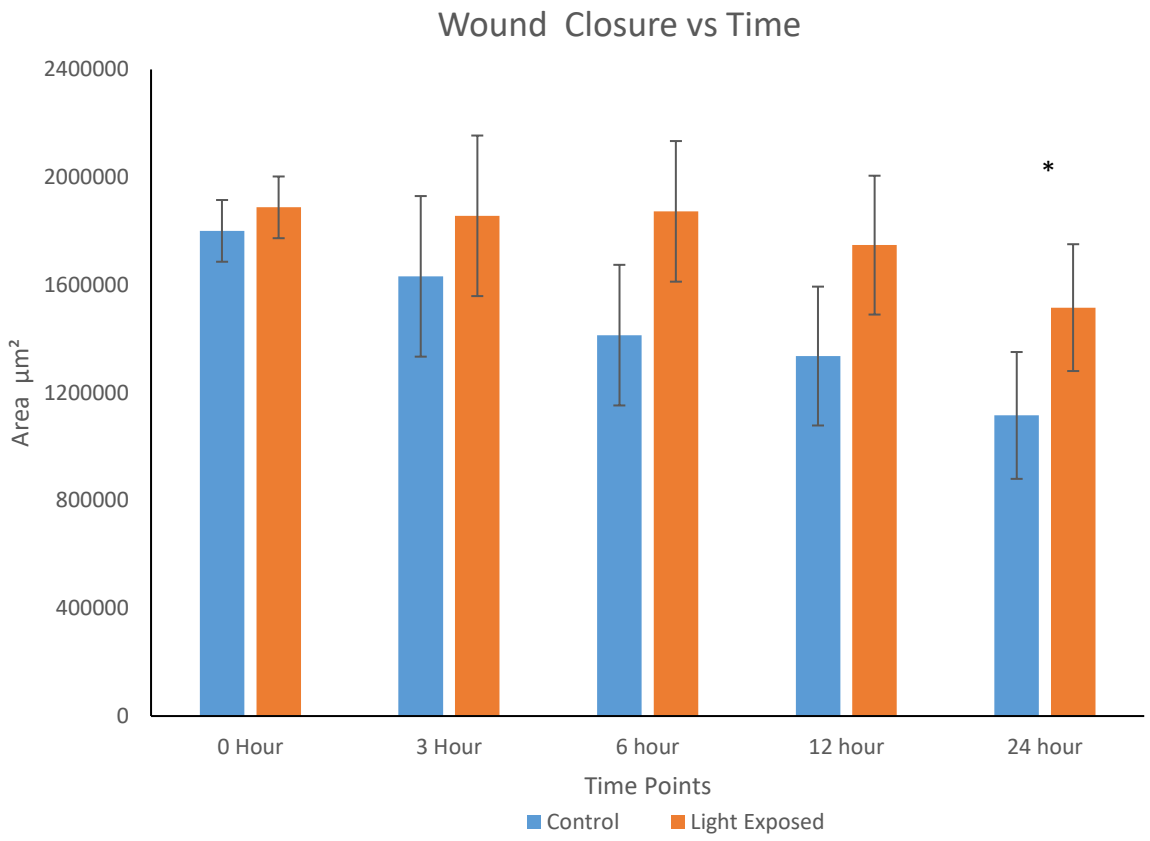

Figure 3-10. The wound area for light exposed and control samples at different time points. The average of the mean area for each time point for each condition is shown (*p = 0.02). When conservative Bonferroni Correction was applied, the statistical difference did not remain significant.

3.3.2.3 *MTT Results*

The light exposure and control samples with scratch were cultured for 24-hour incubation after the light exposure. The cell viability was assessed with MTT at the 24-hour time point. The results in Figure 3-11 represent p3, p4 and p5 cells. A significant difference in cell viability between light exposed and control samples was only observed in p3 cells ($p = 0.04$). The difference was not significant for p4 and p5 cells ($p > 0.05$) which is most likely related to their proliferation rate.

As the cell population doublings rate was slower with the increasing passage number found (as demonstrated in Figure 3-6), slower proliferation was expected with p4 and so p5 compared to p3 cells, which is due to the limited life span of in vitro models using primary cells.

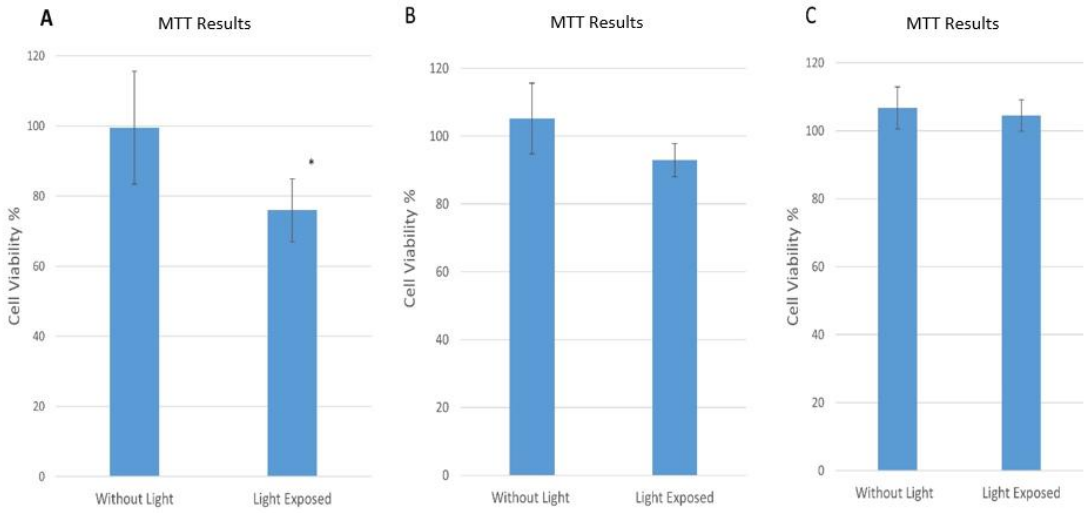

Figure 3-11.MTT results are shown for A) p3 cells and B) p4 and C) p5 cells, indicating the effect of light on cell viability. Cells in the test group were exposed to light for 10 min and both groups were tested with the MTT assay after 24 hours. All absorbance values were normalised to the control. Data represent the percentage of mean viable cells. Student t-test was used to compare the groups (*p = 0.04).

3.3.3 Real World Simulation

In this section, the experimental set up was used with the real world operating microscope. The results of the pilot study were used to improve the in vitro model.

3.3.3.1 *Light Exposure and Light Intensity Measurements*

The light exposure time was kept constant throughout the experiments at 10 minutes. The spectrum of the operating microscope (Zeiss, Oberkochen, Germany) with the correction of daylight is shown in Figure 3-12. The irradiance of the microscope was found as 121 W/m².

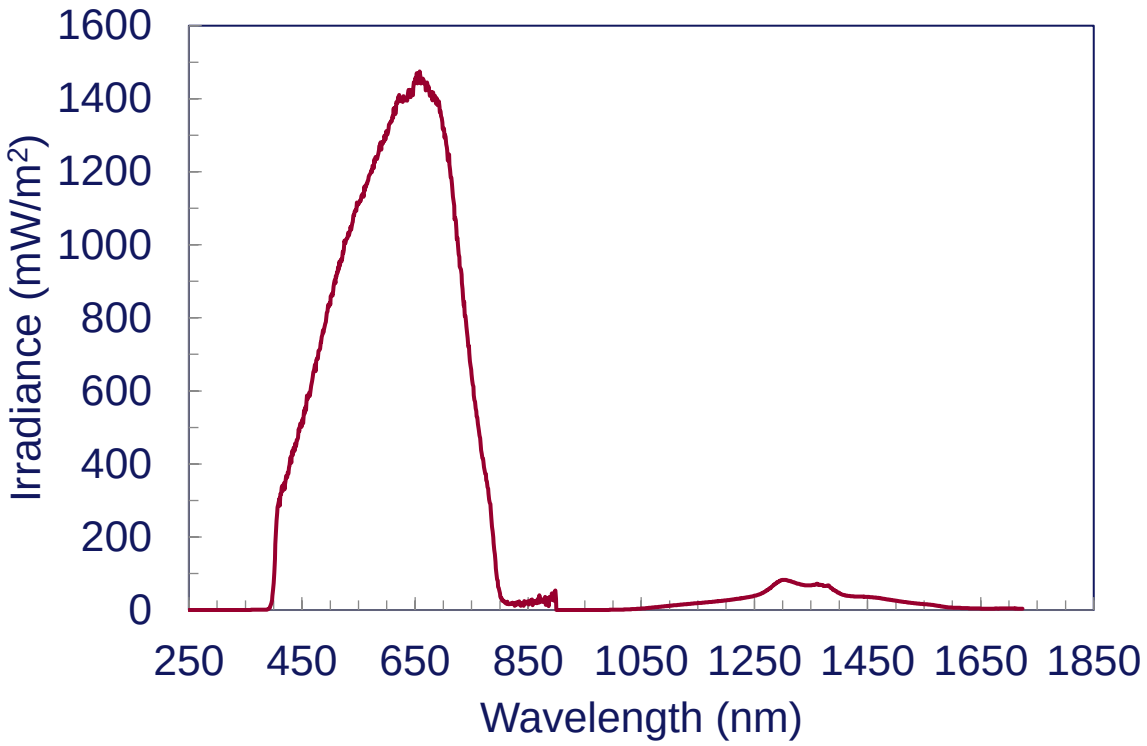

Figure 3-12. Background corrected spectrum from the operating microscope.

The light exposure was performed both on 96 well plates and also petri dishes. One petri dish was used at a time for light exposure. Black 96 well plates were preferred for the experiment. Four wells at a time from different hyperosmolarity were used for each light exposure sample. Half of the 96 well plate was used as the dark samples by covering them with aluminium foil. The distance from the sample to light sources was fixed and kept constant throughout the experiments. The highest intensity was used for all the light exposure experiments to keep the intensity stable and at the same magnitude for each experiment.

3.3.3.2 *Preoperative Dry Eye*

3.3.3.2.1 Cell Viability (Quantitatively)

Before the actual preoperative dry eye model simulation was performed, the effect of hyperosmolar medium alone, hyperosmolar medium with light exposure without a scratch assay were assessed by cell viability in order to assist in interpreting the results later.

Conjunctival fibroblasts cultured in supplemented medium with 328 mOsm/kg, 370 mOsm/kg, 412 mOsm/kg, 480 mOsm/kg osmolarity were initially tested for the viability with 72 hours without any other treatment. There was no significant difference in cell viability between the control, 370 and 412 mOsm/kg medium. When the cells were cultured in 480 mOsm/kg medium, a significant reduction of the cell viability was observed (p=0.02). The viability was measured at this time point in order to interpret the light exposure effect with the preoperative and postoperative experimental model applied later.

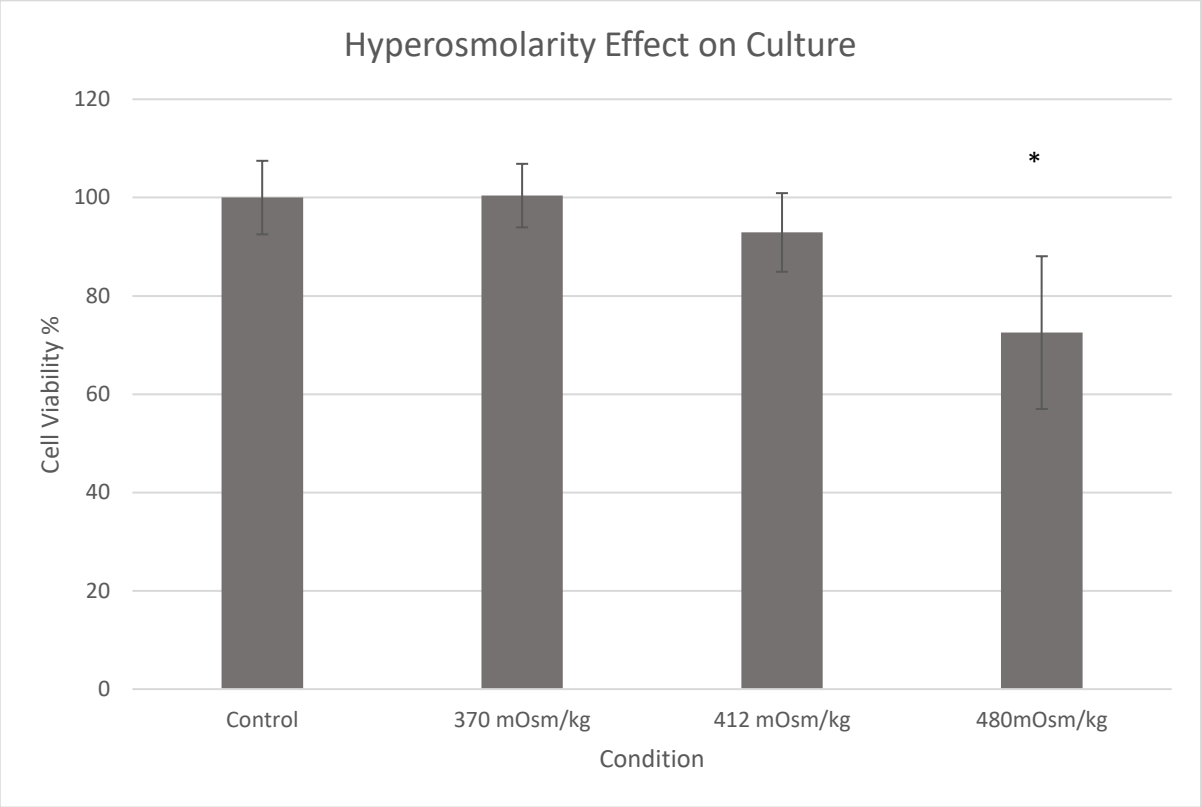

Figure 3-13. Cell viability differences with the different hyperosmolar medium for 72 hours of incubation. MTT results are shown with the different hyperosmolar medium. Statistically significant results were achieved at the 480 mOsm/kg with Student T-test p=0.02. Stars (*) refers to statistically significant reduction with respect to the control values.

Conjunctival fibroblasts were cultured within the different hyperosmolar medium for 48 hours followed by 10 minutes of light exposure. The cells were terminated for their viability to be assessed as shown in Figure 3-14 at the 24-hour time point after light exposure. The light exposure was not found to have an effect on cell viability within the groups.

Statistical differences were observed between both light exposed and dark samples with 412 and 480 mOsm/kg culture conditions compared to the control. This indicates that if there is no conjunctival damage (no scratch), light exposure does not affect cell viability. Only within the culture group of 480 mOsm/kg conditions, there was a significant reduction in light exposed compared to the control.

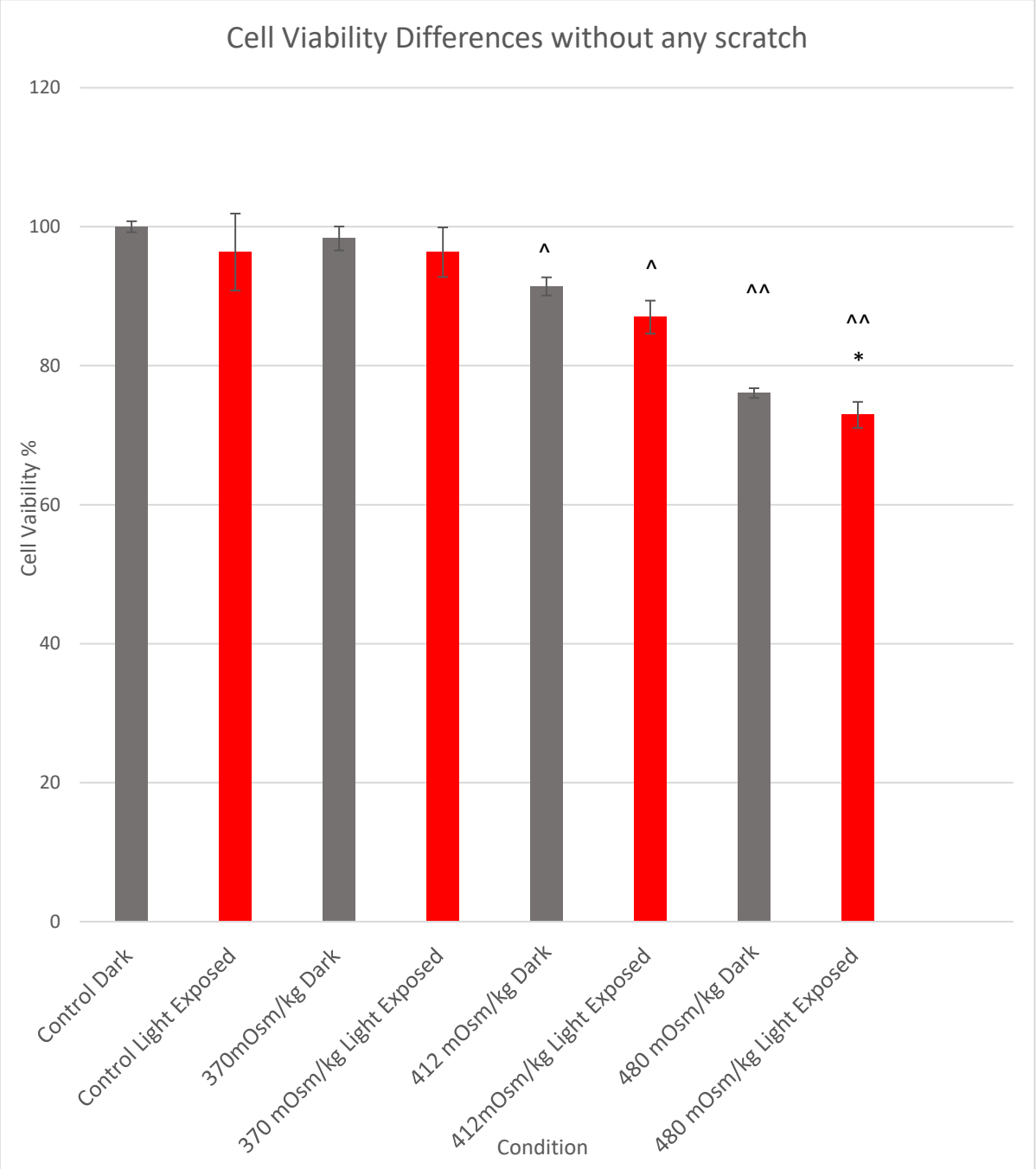

Figure 3-14. Viability differences of the conjunctival fibroblasts cultured in different hyperosmolar medium followed by light exposure without a scratch. The cell viability was measured at 24 hours after 10 minutes of light exposure. Only the 480 mOsm/kg dark and light exposed condition had significantly reduced viability compared to the control dark values. Two-way ANOVA was used to test statistical significance. Stars (*) refer to the statistically significant reduction compared to the same culture conditions but without light exposure (*p<0.05). Carets (^) represent the statistically significant reduction compared to control culture condition but the same exposure. (^ p<0.05, ^^ p<0.01)

Preoperative dry eye simulation was initially assessed by fibroblast viability Figure 3-15.

Scratch assay was performed after the cells were cultured within different hyperosmolar media to mimic the preoperative dry eye. Conjunctival damage was simulated by creating a scratch assay as shown schematically in Figure 3-1. The cell viability differences were assessed after 10 minutes of light exposure at the 24-hour time point. The light exposure caused a reduction within all different culture conditions. Although a significant reduction was only found with 480 mOsm/kg, due to the higher fluctuations in the samples, the pattern of light-exposed samples can be seen. With 480 mOsm/kg culture conditions, cell viability almost reduced by 70%.

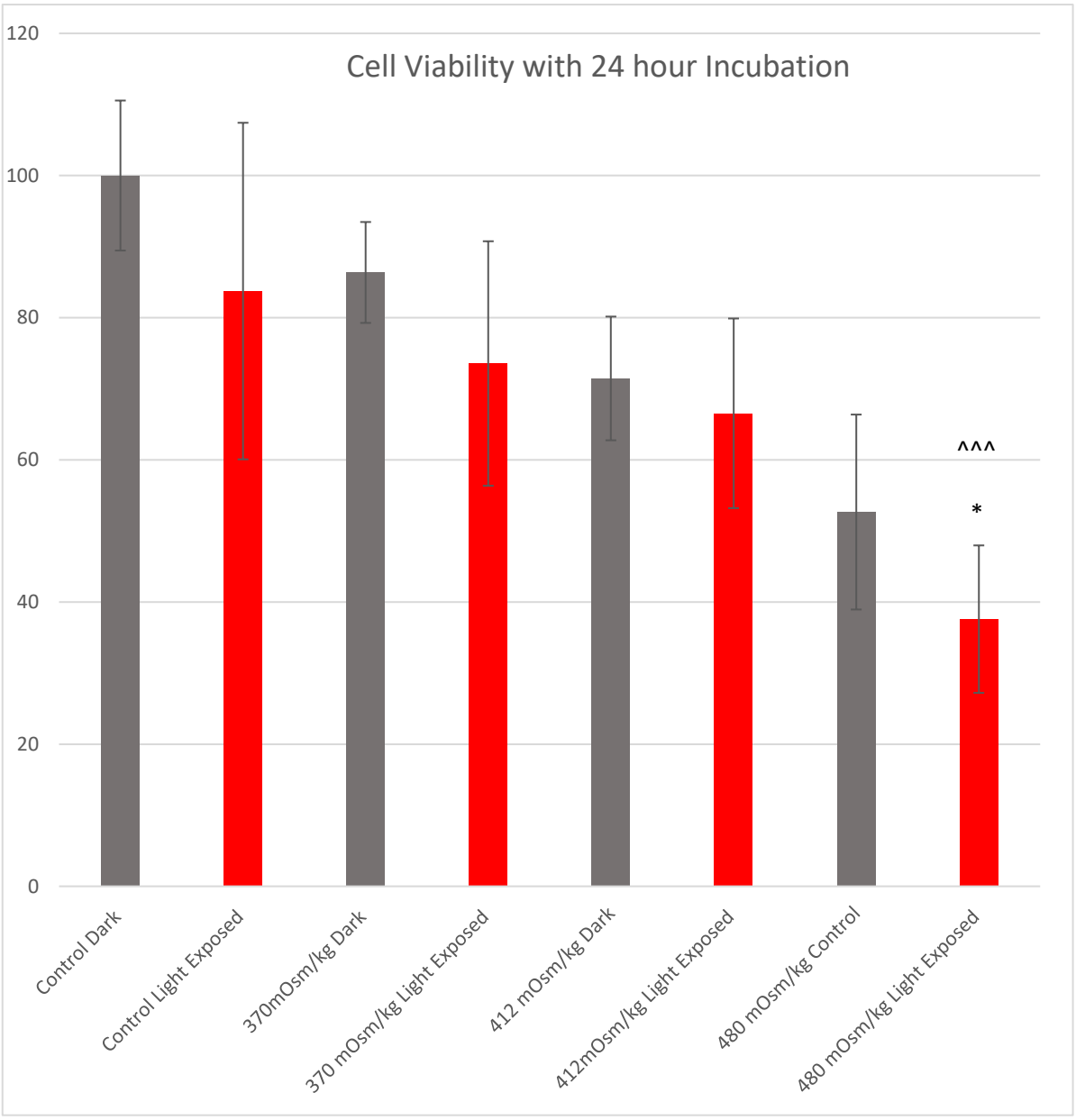

Figure 3-15 Cell viability differences with scratch assay followed by light exposure within the hyperosmolar medium. Preoperative dry eye simulation was carried out with initially with hyperosmolar medium change 48 hours before the scratch assay and light exposure. Scratch assay was followed by 10 minutes of light exposure. Cells were terminated at 24-hour incubation after light exposure. Two-way ANOVA was performed to test for the difference. Stars (*) represent the differences with the correspondent dark condition of the same culture medium and carets (^) indicates the differences between the culture medium differences within light exposed samples. * $p<0.05$, ^^^ $p<0.001$.

3.3.3.2.2 Wound Healing

Wound healing was assessed with images obtained by light microscopy seen in Figure 3-16 and fluorescence microscopy in Figure 3-17 for the preoperative dry eye model. Preoperative dry eye simulation was performed after 48 hours' exposure to the hyperosmolar medium prior to the scratch assay. The light exposure was performed after the scratch assay was created. All the images were taken after 24 hours of incubation following the light exposure.

Cells cultured in a medium with 370 mOsm/kg hyperosmolarity had almost the same wound area as the cells in the control medium conditions. On the other hand, higher hyperosmolar media; 412 mOsm/kg and 480 mOsm/kg were found to have greater wound area as opposed to control medium conditions.

Light exposure was found to delay the wound healing when the cells were cultured in 370 mOsm, 412 mOsm/kg and 480 mOsm/kg compared to dark conditions with same medium conditions. Conversely, delayed wound healing was more obvious with the 412 mOsm/kg and 480 mOsm/kg culture conditions. Within the control samples, light exposure caused delayed wound healing compared to the dark samples, but the differences were not as dramatic as in the other hyperosmolar medium conditions.

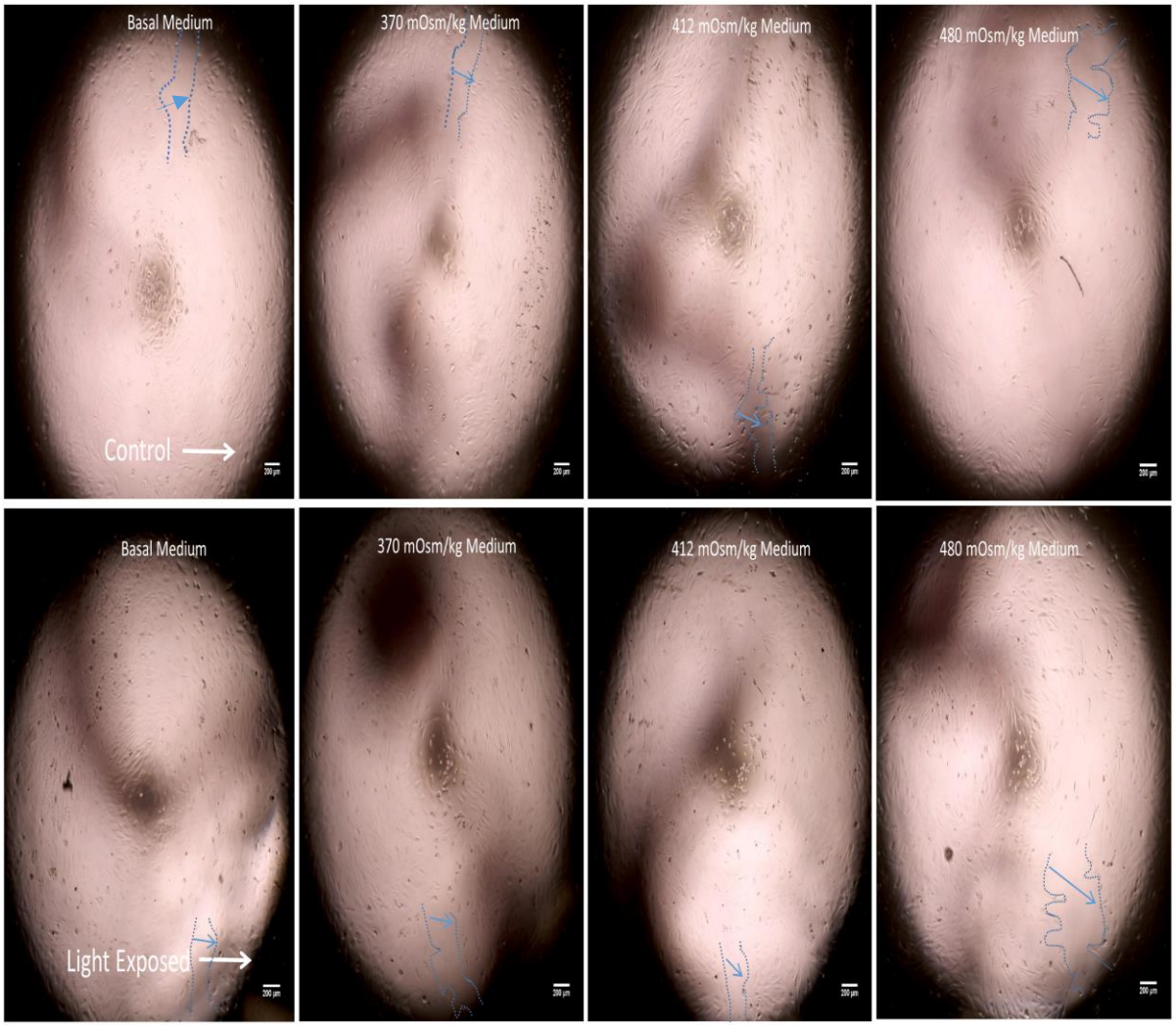

Figure 3-16. The scratch assay after 24 hours by light microscopy. 10 minutes of light exposure was carried out immediately after the scratch was created. The slower migration of cells was observed at the higher concentration of the hyperosmolarity culture with light irradiation. The blue dotted lines and the arrows indicate the scratch area which was created 24 hours earlier.

The samples from Figure 3-16 were stained with the Live/Dead assay (Figure 3-17). Although it was not possible to observe the whole well due to the lack of field of view of the fluorescence microscope, the scratch area can be clearly observed.

Slower wound healing can be seen with light exposure. With increasing osmolarity, the wound healing was much slower compared to the control medium conditions. Light exposure also delayed the wound healing, particularly with the 412 mOsm/kg and 480 mOsm/kg samples. With 370 mOsm/kg and control samples, the difference in the wound was not that striking compared to 412 mOsm/kg and 480 mOsm/kg. Labelled dead and live cells can be seen throughout the samples. The cells were predominantly labelled as live. However, since it was not possible to observe the whole well, the live/dead range was not taken into consideration.

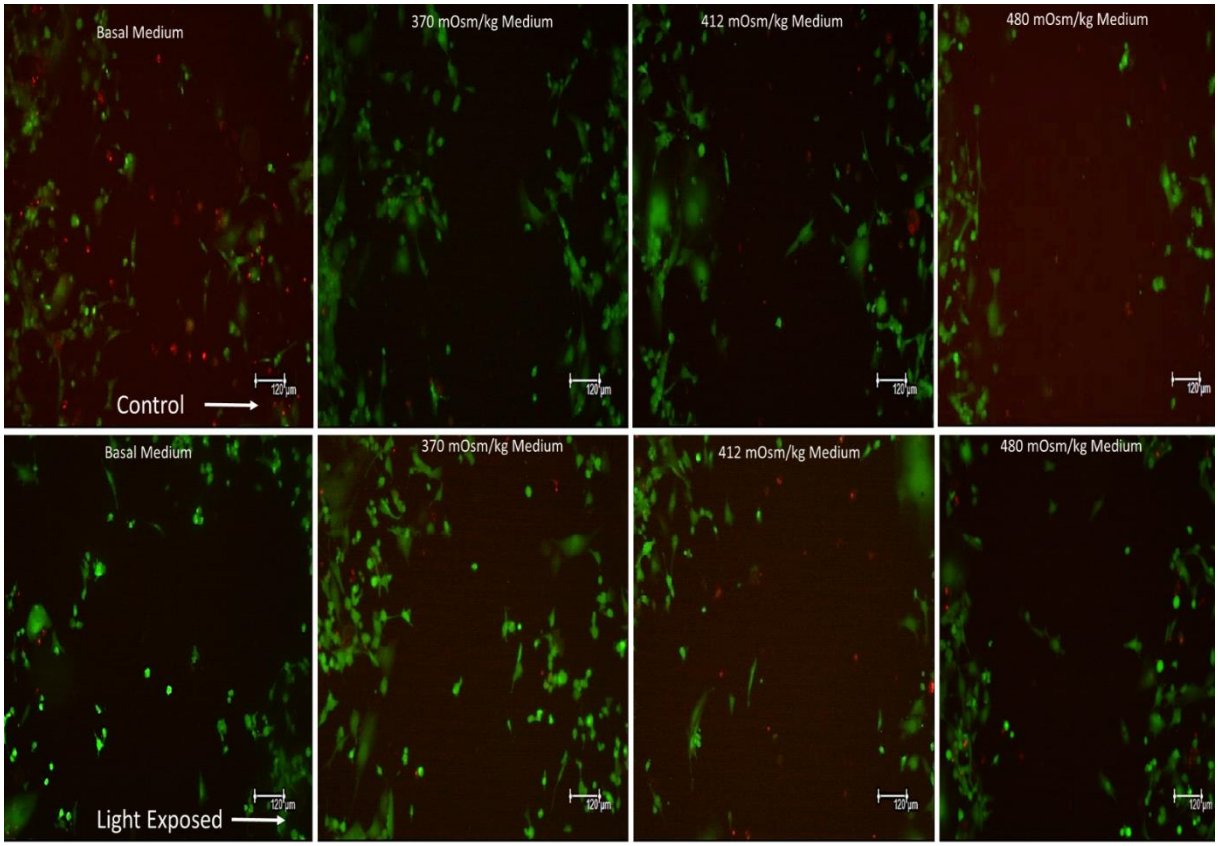

Figure 3-17. Live/Dead assay after 24 hours of light exposure with the scratch assay. Hyperosmolarity exposure after the scratch and light exposure was used to mimic the post-operative dry eye. The wound healing differences were slower with the higher osmolarity and also light exposure.

3.3.3.2.3 Cell Size Differences

Cell size was analysed with different samples in this preoperative dry eye model, outlined in Figure 3-18. The assessment was performed at 24 hours after 10 minutes of light exposure as for the rest of the analysis. However, there was no significant difference in cell size with increasing osmolarity or with light exposure.

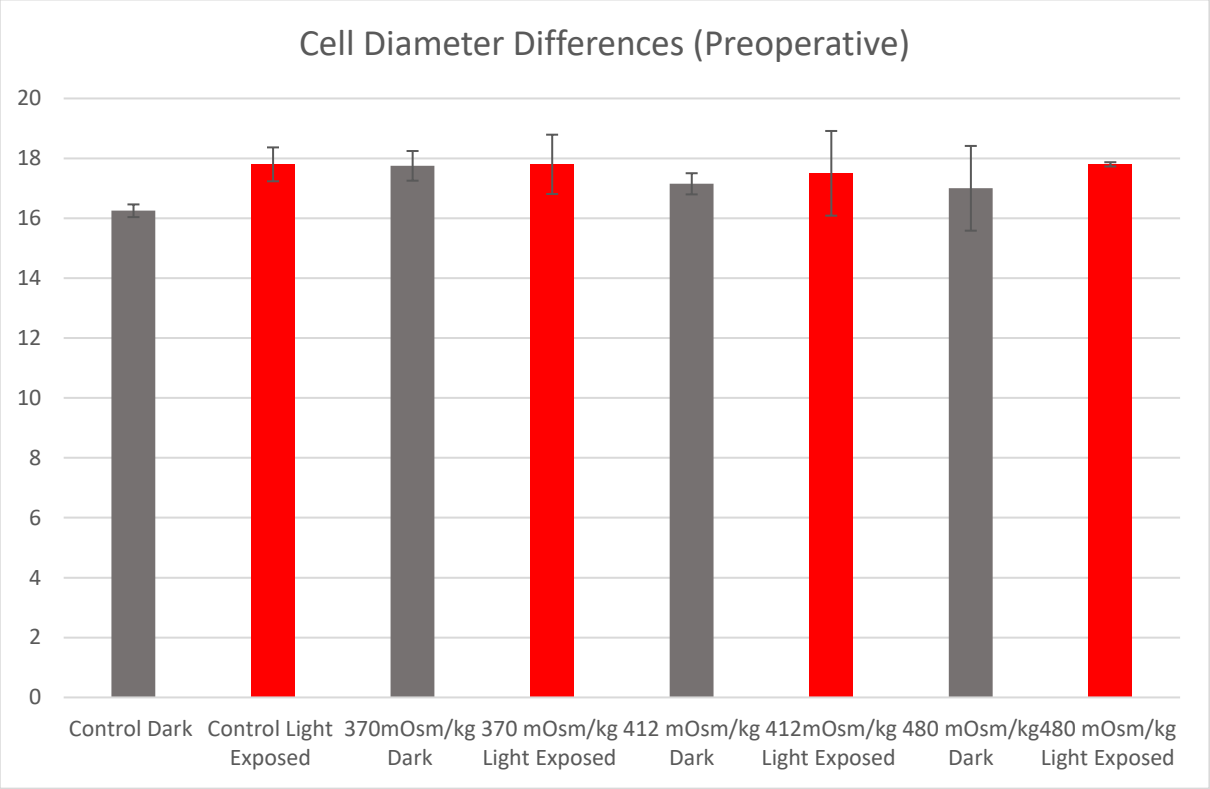

Figure 3-18. Differences in cell diameter for the in vitro preoperative dry eye model. Cells were exposed to a hyperosmolar medium for 48 hours before scratch assay and light exposure. The analysis was carried out at 24-hour time point after light exposure. No significant difference was found between the samples.

3.3.3.3 *Annexin V Assay*

The annexin V assay was performed to detect the cells that express phosphatidylserine on the cell surface which is a characteristic of the apoptotic cell, seen in Figure 3-19. The gates were set up according to the control (dark) results and the rest of the gating channel were normalised according to the control values. Healthy cells were visible in the lower left quadrant labelled as Annexin V-/PI- while early apoptotic cells were seen in the lower right quadrant labelled as Annexin+/PI-. The late apoptotic cells were represented in the upper right stained with Annexin V+/PI+ whereas necrotic cells were observed in upper left quadrant labelled as Annexin V-/PI+. The apoptotic cells were counted as a percentage of Annexin V+/PI and Annexin V+/PI+, while necrotic cells were counted cells stained as Annexin V-/PI+.

There were no significant changes in the cells without any light exposure in terms of annexin V level except the 480 mOsm/kg condition compared to control. Although the trend indicates that there is a

higher tendency in the light exposed samples, there was no significant difference between dark and light exposed samples within each group except the 412 mOsm/kg medium condition.

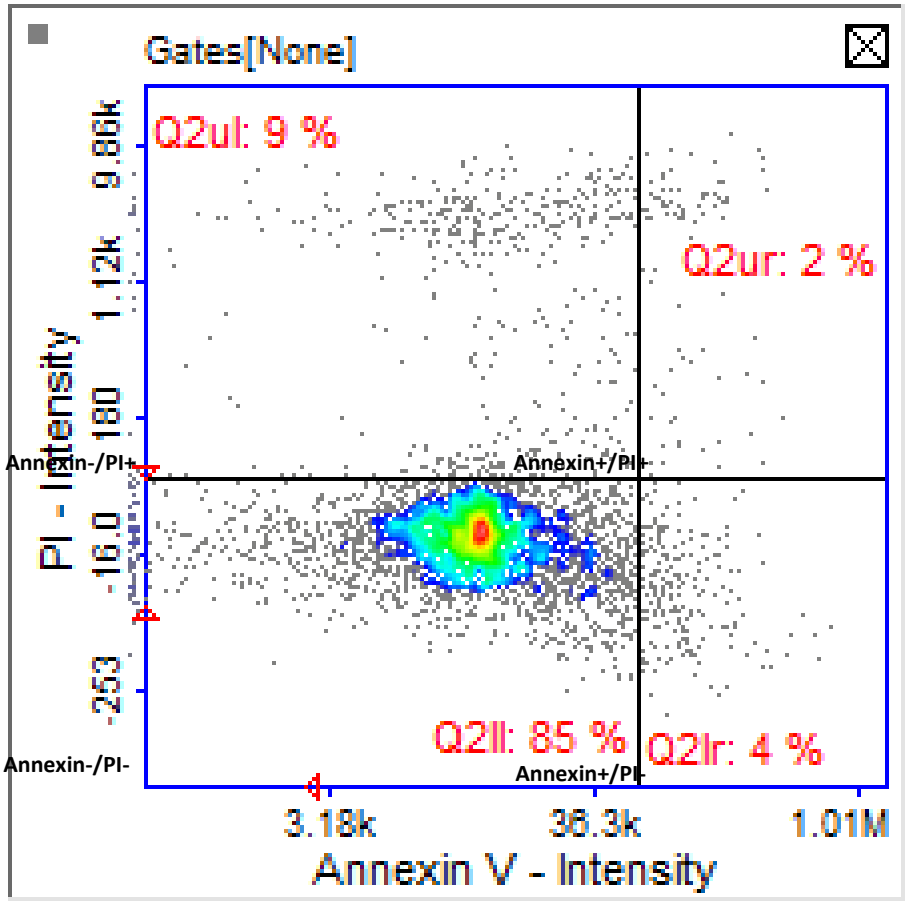

Figure 3-19. Example of gating for annexin V assay. Following scratch assay and light exposure for the preoperative dry eye model, the apoptosis rate was detected at the 24-hour time point. The healthy cells were represented in Annexin-/PI- in the lower left quadrant while pre-apoptotic cells were observed in Annexin+/PI- in the lower right quadrant. Late apoptotic cells were represented in the upper right quadrant as Annexin-/PI+. Cell death was observed in upper left quadrant labelled as Annexin-/PI+.

Each gate was calculated individually to observe the differences in the cell status in terms of its health within each group. Firstly, healthy cells stained with neither Annexin and PI were shown in Figure 3-20. Statistical significance was only observed with the 480 mOsm/kg samples (with and without the light exposure), compared to different culture medium conditions.

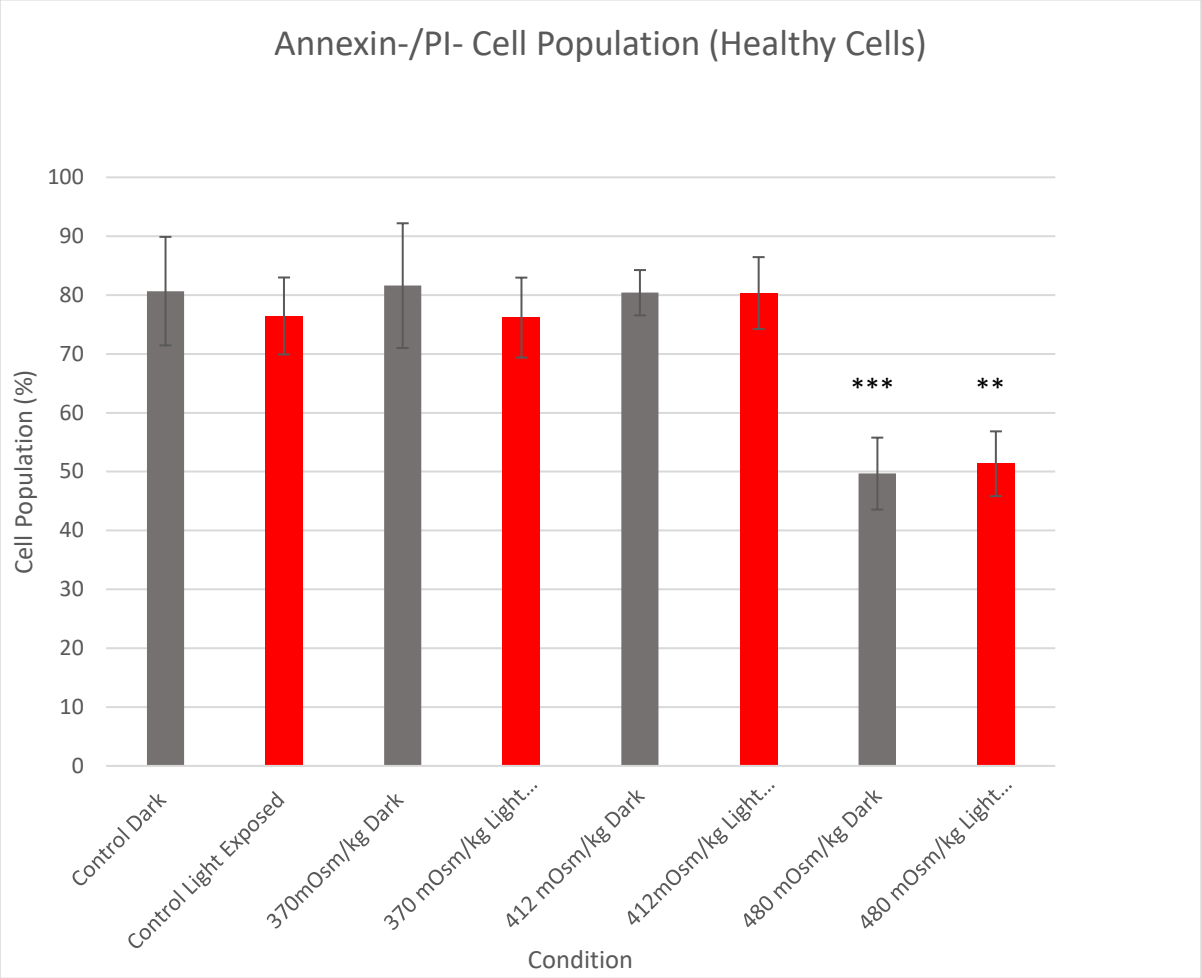

Figure 3-20. The cell population in the Annexin-/PI- gate which is considered as healthy cells. Statistical significance was found with the 480 mOsm/kg culture conditions with and without light exposure compared to control. Stars (*) represent the statistical significance compared to culture medium differences (p<0.01, ***p<0.001).**

Pre-apoptotic cells stained with Annexin+/PI- were observed and shown in Figure 3-21. Elevation of the percentage of cells was found to be statistically significant in the culture conditions with 480 mOsm/kg compared to other culture medium conditions. Light exposure was not found to cause any effect.

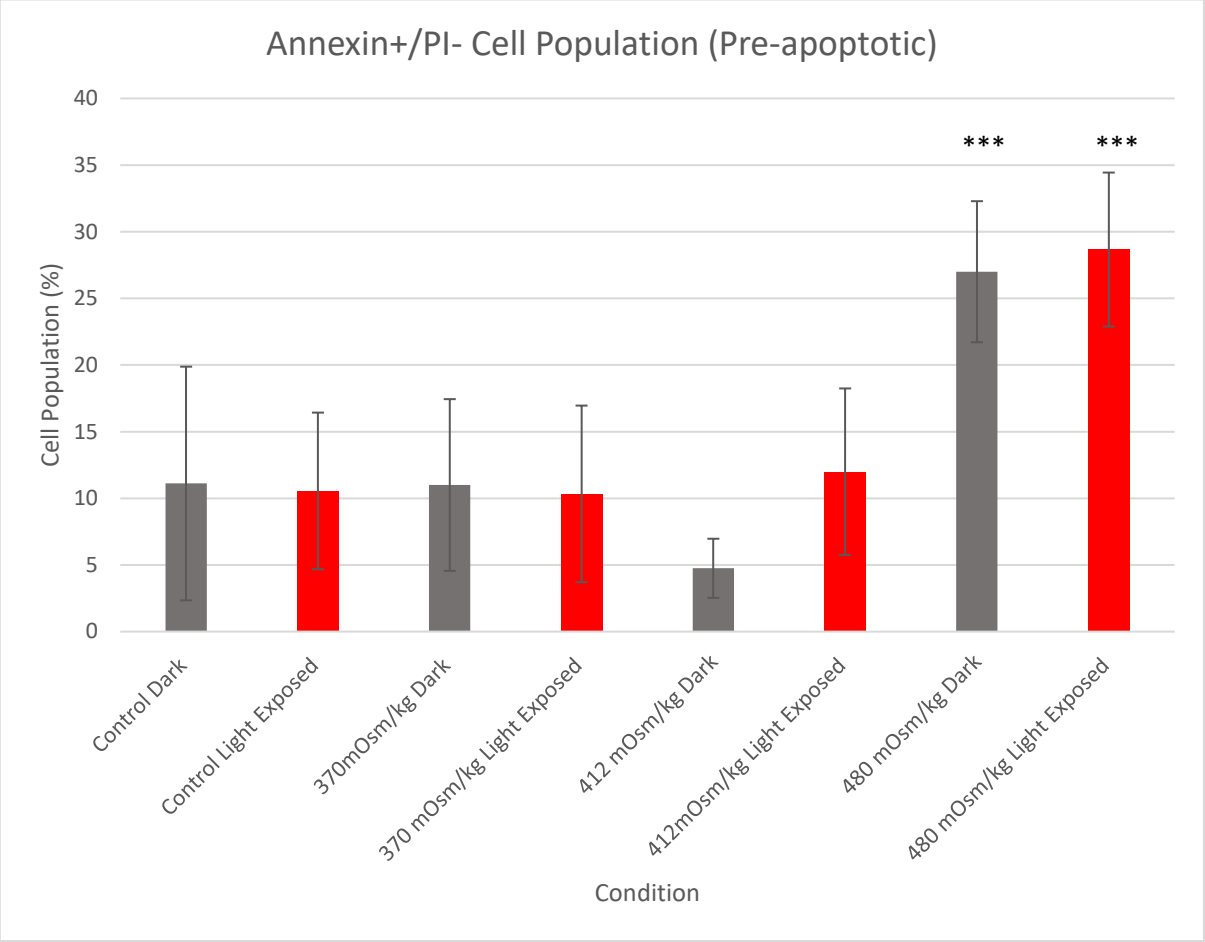

Figure 3-21. The cell population with pre-apoptotic condition stained as Annexin+/PI-. Statistical significance was found with 480 mOsm/kg culture conditions with and without light exposure compared to control with two-way ANOVA. Stars (*) represent the difference compared to the culture medium (*p<0.001).**

Late-apoptotic cells which were stained with Annexin+/PI+ are shown in Figure 3-22. A statistically significant increase was only found with the 480 mOsm/kg condition, with and without light exposure compared to the other culture medium conditions.

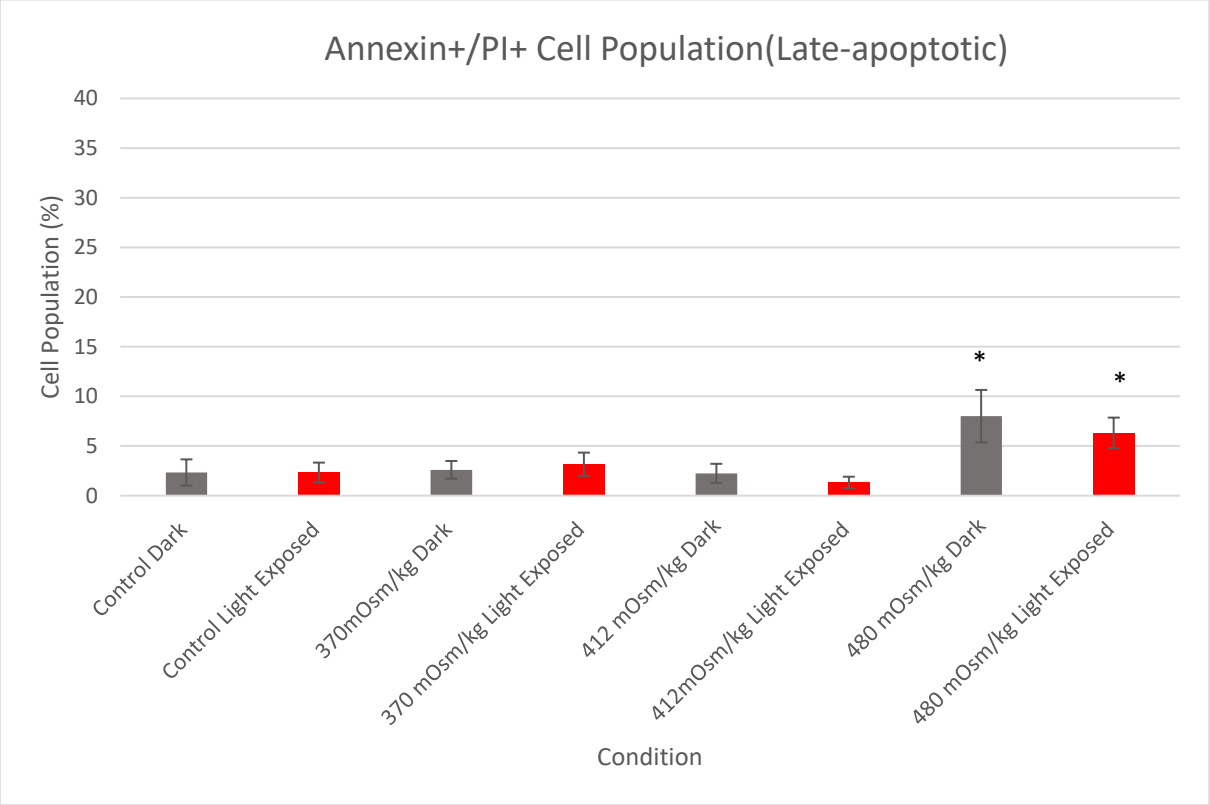

Figure 3-22. The cell population with late apoptotic condition stained as Annexin+/PI+. Stars (*) represent significant difference compared to other culture media conditions (*p<0.05).

The percentage of necrotic cells are quite low compared to the rest of channels with the Annexin V staining, are presented in Figure 3-23. Therefore, it can be confirmed that most of the cells were in either a healthy state or an apoptotic state.

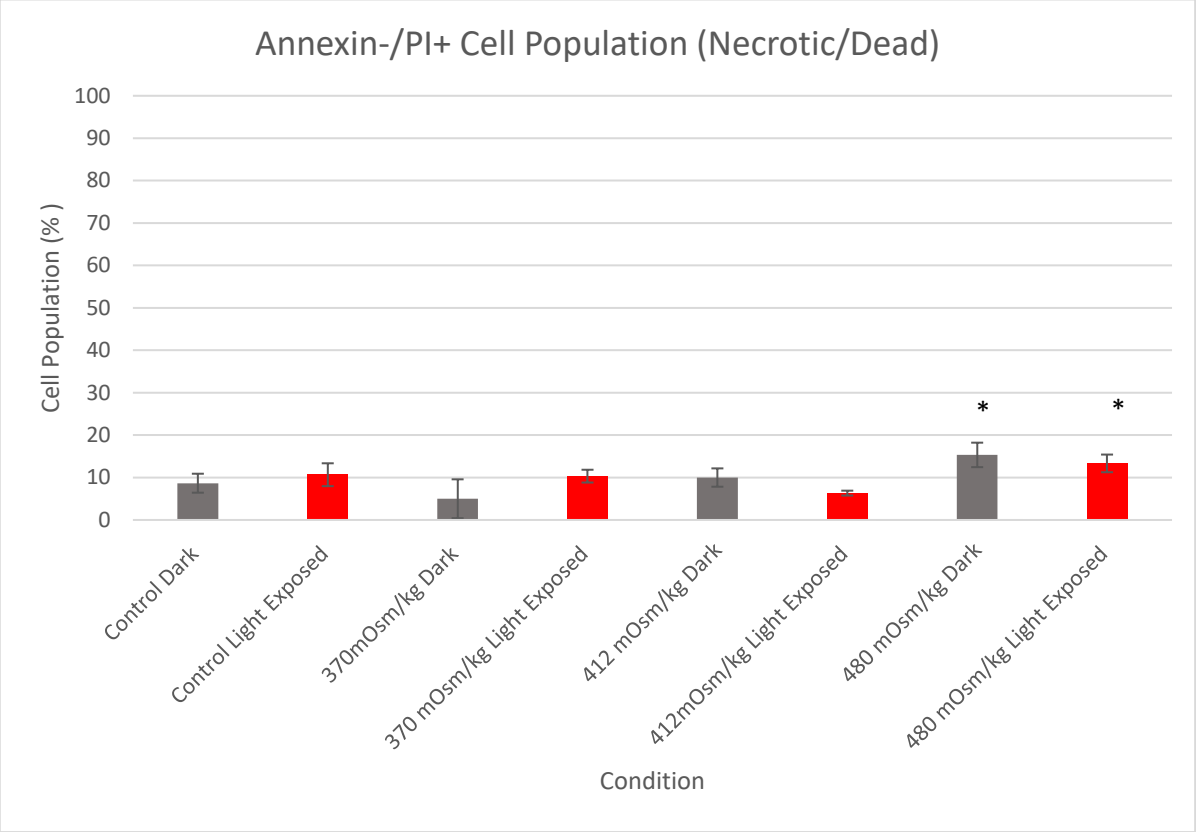

Figure 3-23. The necrotic/dead cell population stained with Annexin-/PI+. Increase in 480 mOsm/kg conditions was statistically significant compared to other medium conditions. Stars (*) represents the statistically significant difference compared to other culture media conditions (*p<0.05).

Annexin+ cells were combined to determine the apoptosis rate between the different conditions. The highest apoptosis rate was found with the 480 mOsm/kg conditions compared to different culture conditions with and without light exposure. No difference was observed within light-exposed samples cultured in 480 mOsm/kg, 370 mOsm/kg, and control samples. The control and 370 mOsm/kg medium conditions behaved the same for all the Annexin V/PI staining for each combination. However, there was a statistically significant increase in apoptosis rate with light exposure at the 412 mOsm/kg culture conditions compared to no exposure as control.

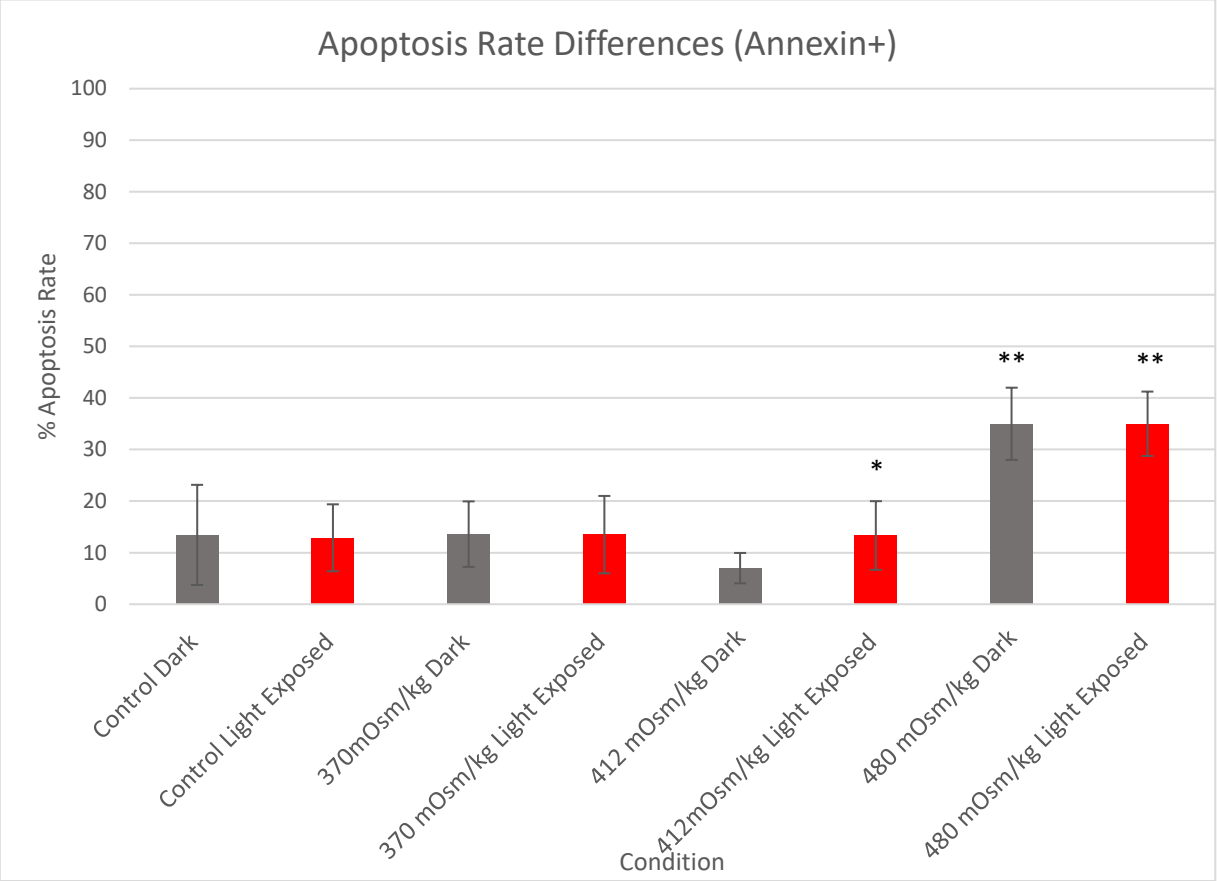

Figure 3-24. The apoptosis rate of cells with the preoperative dry eye model. Cells with stained with Cells stained with Annexin + were indicated within different culture/exposure conditions. Increase in apoptosis rate with 480 mOsm/kg culture conditions was found to be significant. Stars (*) represent a statistical difference compared to other culture medium conditions (* p<0.05, **p<0.01).

3.3.3.4 IL-6 Levels

IL-6 secretion as a pro-inflammatory cytokine was detected to observe the inflammation by using porcine IL-6 ELISA kit, shown in Figure 3-25 with the preoperative dry eye model at the 24 hour time point. Light exposure was found to cause elevation of IL-6 levels compared to the same culture conditions (but without light); this occurred in the control 370 mOsm/kg and 412 mOsm/kg samples but not the 480 mOsm/kg medium. The cells cultured within 480 mOsm/kg medium did not show any statistically significant difference between light exposed and cultured in the dark conditions. This might be due to the less viable cells in the light-exposed cells in 480 mOsm/kg culture conditions, therefore, the secretion of IL-6 into their medium could be not representative of the inflammation state as viable cells were already shown reduced at this condition. Even though the error bars were quite large in the

culture conditions with hyperosmolarity of 412 mOsm/kg, increased IL-6 levels were observed with light exposure as in control, 370 and 412 mOsm/kg culture conditions.

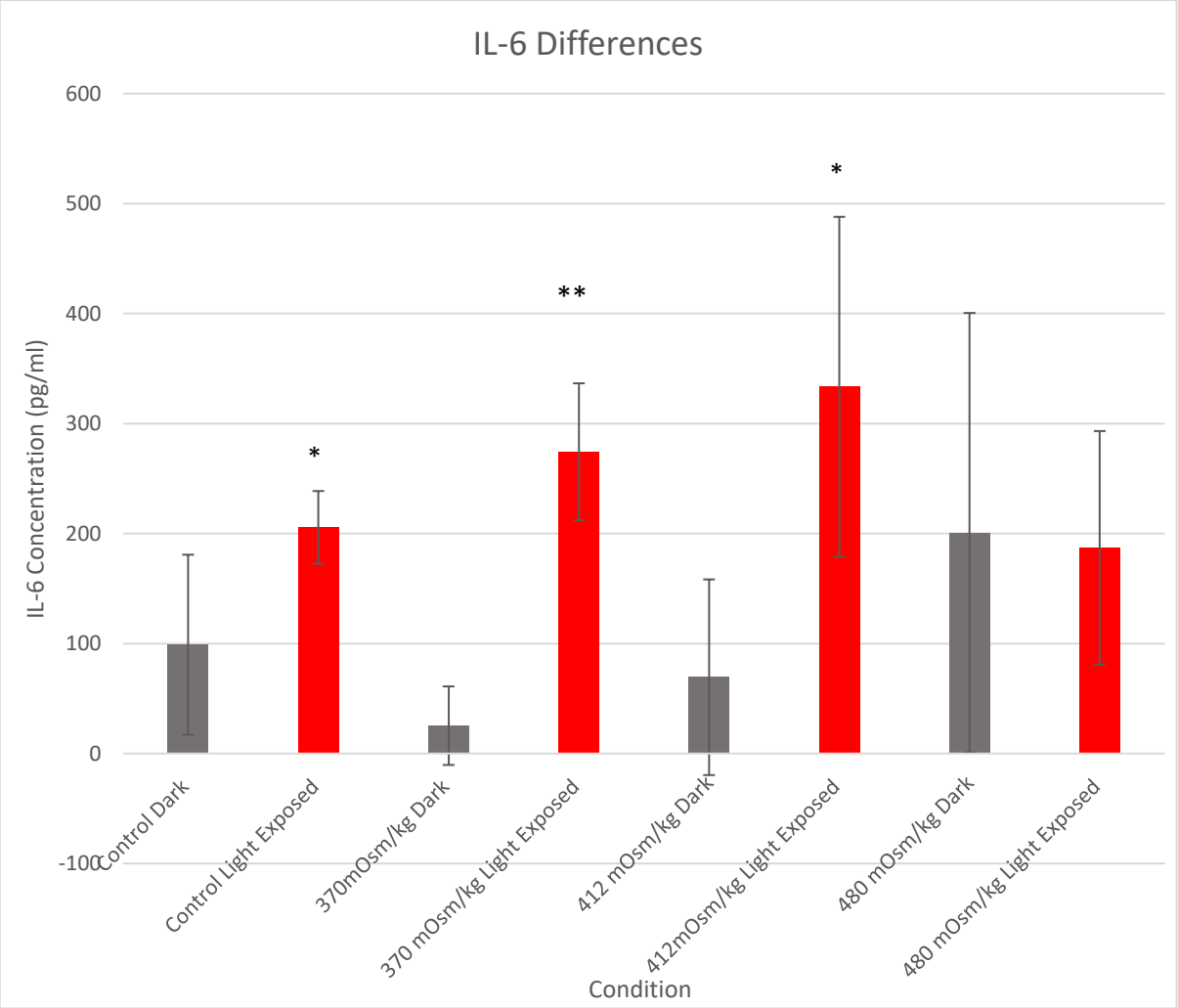

Figure 3-25. The concentration of IL-6 levels in the cell culture medium was shown with different conditions at the 24-hour time point after light exposure. Stars (*) refer to statistically significant increase compared to the same culture condition but without light exposure (dark). (*p<0.05, **p<0.001).

3.3.3.5 *Postoperative Dry Eye Model*

When the cells reached 70% confluency, the scratch assay was performed. Immediately after the scratch assay, light exposure was performed for 10 minutes. Post-operative dry eye was simulated by changing the culture medium to hyperosmolar medium after light exposure, schematically shown in Figure 3 2. After 24 hours of incubation, cells were terminated to have their viability analysed as shown in Figure 3-26. No statistically significant difference was found with light exposure or culture medium differences.

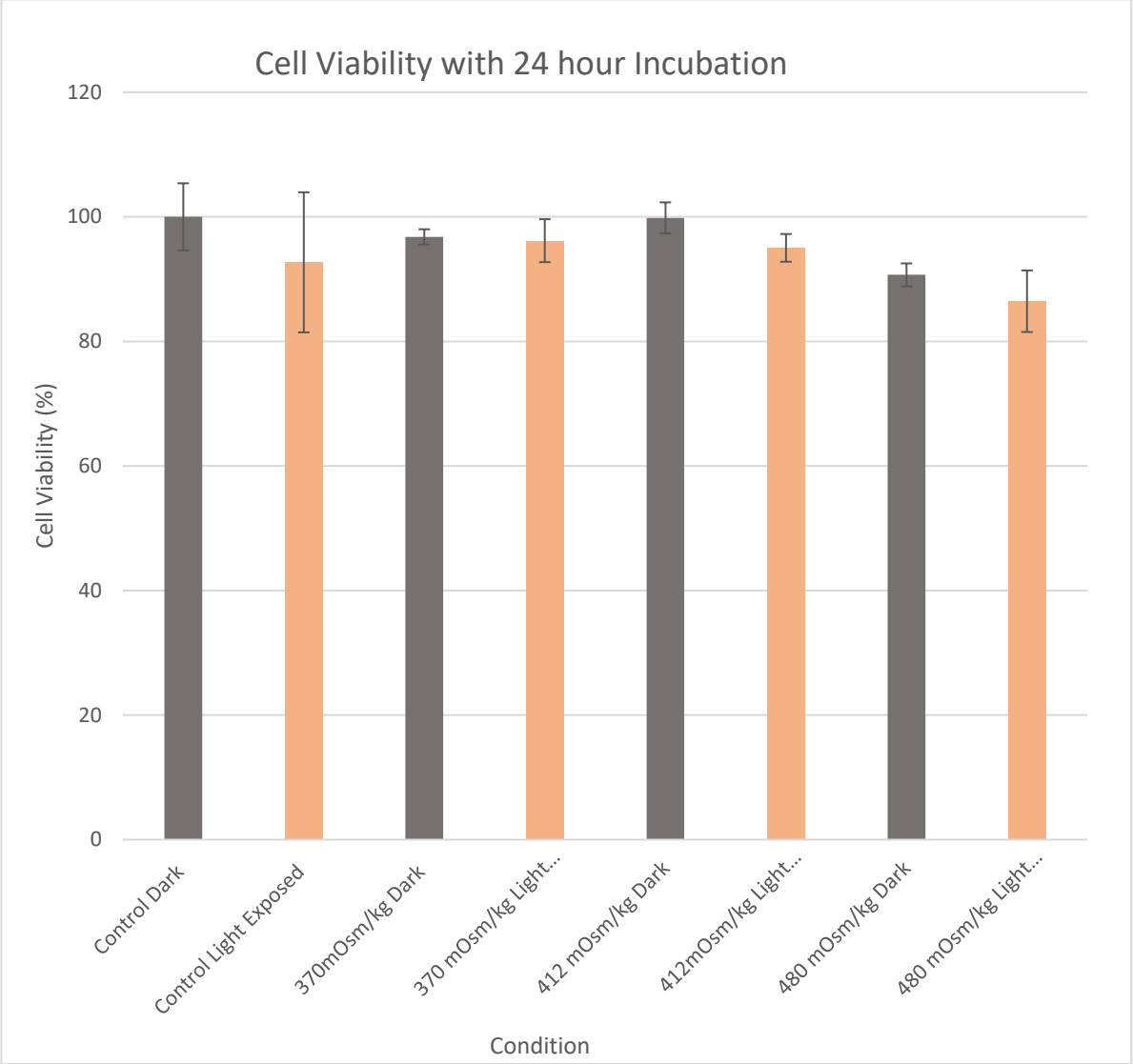

Figure 3-26. Cell viability differences when the cells were incubated for 24 hours after scratch assay and 10 minutes of light exposure. Postoperative dry eye simulation was performed initially with scratch assay followed with 10 minutes of light exposure. After light exposure, the cell culture medium was changed to hyperosmolar condition. Cells were terminated after 24 hours of incubation after light exposure. Although a small reduction in cell viability can be seen with light exposure in each culture condition, no statistical difference was observed between samples.

With the postoperative dry eye model, after 48 hours of incubation, cells were terminated to analyse their viability, to assess whether the longer incubation period makes any difference in terms of viability. No statistical difference was observed with light exposure or culture medium differences (shown in Figure 3 27). In fact, the cell viability differences were found to be less than the viability differences when the cells were incubated for 24-hours

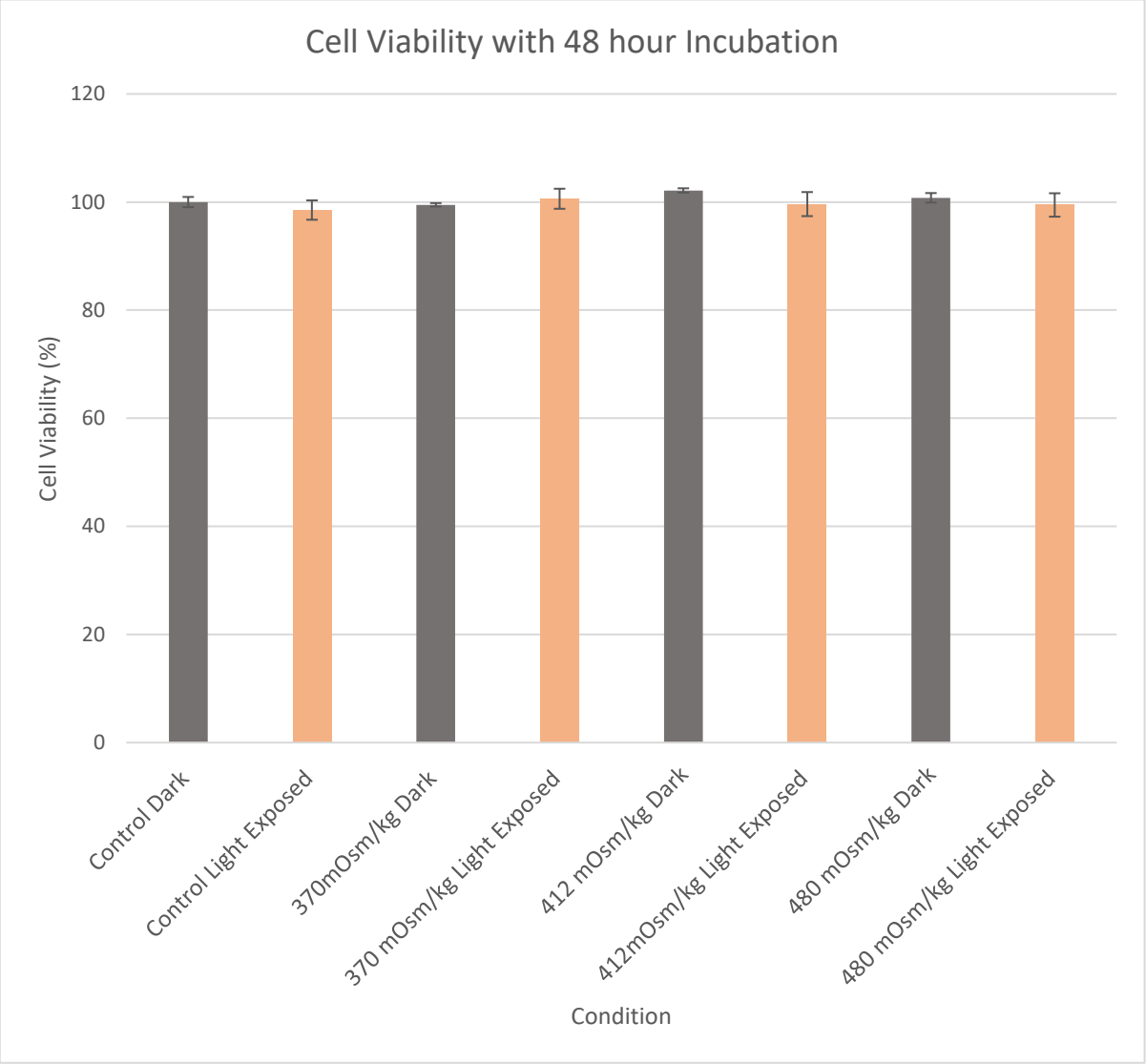

Figure 3-27. Cell viability differences when the cells were incubated for 48 hours after scratch assay and light exposure. Postoperative dry eye simulation was carried out with scratch assay and light exposure respectively. Cells were terminated at 48-hour incubation after light exposure. No statistical difference was observed between the samples.

Cell diameter differences was observed with the post-operative dry eye model; no statistical change was observed, presented in Figure 3-28.

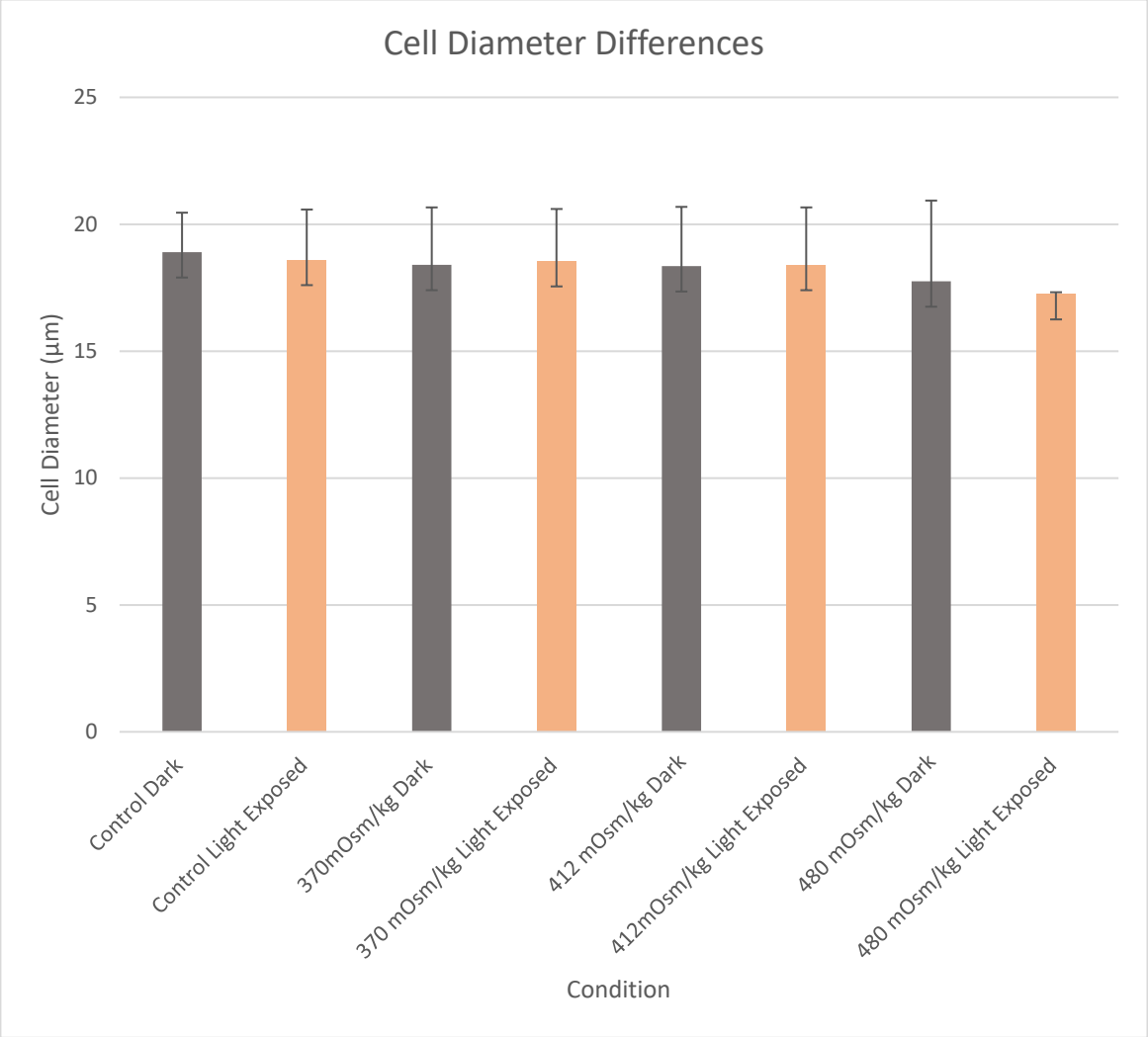

Figure 3-28. Cell diameter differences with the postoperative dry eye model. No significant difference was found between the cell diameter.

3.4 Discussion

The multifactorial nature of dry eye is still not fully understood [1]. Ophthalmic surgery may have the potential to temporarily induce or worsen dry eye, typically during the short postoperative period [110,130,149,265]. Cataract surgery, for instance, might lead to disruption of homeostasis of the ocular surface due to the use of a speculum, surgical incisions and use of pre-operative and/or intra-operative pharmaceutical agents. Light exposure from the operating light microscope can also have an effect on the ocular surface, which is already 'stressed' under the conditions of surgery, possibly contributing to the development of signs and symptoms of dry eye.

The detrimental effect of phototoxicity on the retina due to various sources of light has been well documented in the literature [257,259,260]. More recent attention has been focused on the effects of blue light on the ocular surface [116,153,168,266–269] however, there are only a few studies that have looked at the impact of the full spectrum of light emitted from an operating microscope onto the ocular surface [116,153,175]. It is still not clear precisely how the ocular surface is affected by light exposure. Retinal damage due to light exposure is known to occur through photomechanical, photothermal and photochemical mechanisms, photochemical damage being the most common mechanism of photic changes due to operating microscopes [257,259,260]. The photo-chemical disruption occurs from the generation of free radicals or reactive oxygen species which can disrupt the poly-unsaturated fatty acids located on cellular membranes [257]. Hwang and Kim [116] have recently shown the effects of the operating light microscope on the rabbit ocular surface; they found an elevation of IL-1β levels in rabbits' tears, which is thought to be due to the generation of reactive oxygen species as described above. The authors of this study suggest that the effect is most likely photochemical, as the photothermal effect was likely to be negligible due to the short period of light exposure. In this study, the effect of light exposure, therefore, is considered as photochemical based on the same rationale. A light exposure time of 10 minutes was used throughout the chapter. Although this is shorter than other similar studies in the literature [116,168] since routine cataract surgery usually takes around 10 to 15 minutes [270]. Exposure time of 10 minutes was used as it may be considered to be at the lower end of the light exposure time that a patient may experience during a typical cataract procedure.

When measuring the spectra of the microscopes, a small discontinuity was observed in the spectrum for the laboratory microscope at 410 nm (Figure 3-7). This is the point at which the results from the UV and visible spectrometers are stitched together and it is believed that this reflects the technical difficulties mounting the integrating sphere in the limited space available with the laboratory microscope. There is likely to be a slight movement of the sphere in the interval whilst the spectrometers were switched since the spectra were achieved with a UV, visible light and infrared spectrometer. However, any difference was marginal and should not affect the results.

Operating microscope spectrum instead truncates sharply at the UV/visible boundary which is believed to reflect the presence of a long pass cut-off filter in front of the light source to remove the UV emissions. Therefore, it is believed that there will be slightly more UV-A in the laboratory microscope compared to the operating microscope since neither microscope shows emission in the infrared even though the operating microscope has a much sharper cut-off which is due to another cut-off filter to block the infrared radiation. Even though both light sources delayed wound healing and caused viability reduction on conjunctival cells within the scratch model, cell viability was affected more with light exposure from the laboratory microscope. More than a 20% reduction was found with the laboratory microscope, but no significant reduction was found with light exposure from the operating microscope with control medium conditions. The difference can be explained with the higher UV-A observed with the laboratory microscope spectrometer since it is already known that UV-A can adversely affect conjunctival fibroblasts [190].

In addition, cell viability was reduced significantly when using cells within earlier passage number (p3), but not in p4 or p5 cells with a laboratory microscope. Therefore, the earlier passage numbers were used for the later experiments (up to p4). This apparent inconsistency in cellular behaviour might be due to the ageing of the cells with the increasing passage number which was investigated with population doublings' rate as seen in Figure 3-6. It has been known that cellular health declines and so may the cell proliferation rate, as the passage number of cells increases. [21,22]. Therefore, the cellular response to light exposure does not necessarily give the same result with the higher passage number. This study found that cell viability was not affected by the light exposure with higher passage number which can be due to the slower growth rate which is observed in Figure 3-6 and most likely a higher resistance to stress [271]. Although a considerable amount of the literature supports the lower resistance with higher passage number predominantly, there are also studies indicating the resistance to apoptosis and cell death with higher passage number of the cells [271].

In this experimental setup, using a scratch assay was utilised to simulate the mechanical trauma of cataract surgery, caused by incisions and the use of a speculum preventing blinking leading to cell desiccation. Light exposure from both microscopes was found to slow down the wound healing

however further studies are required to confirm quantitatively if there is a difference in wound healing response as opposed to different light sources. In terms of cell viability, without a scratch, the cell viability was not affected by light exposure from the operating microscope. This can be explicated that in the case of no damage (in this case it is the surgery), light exposure from the operating microscope would not be detrimental in terms of cell viability.

Dry eye pathology was simulated with the hyperosmolar medium by culturing the conjunctival fibroblasts within. In dry eye patients, hyperosmolar tears continuously bathe ocular surface cells. However, in vitro models can only mimic the condition for a shorter amount of time. Therefore, most of the in vitro studies have used higher hyperosmolar stress levels to accelerate the stress response experienced in vivo dry eye conditions (>316 mOsm) [210,213,215]. Moreover, resistance to the higher osmolarity in vitro conditions was already observed with ocular surface cells, therefore, it is not unusual to use higher hyperosmolarity to mimic the stress in vivo [168,213,272]. For all these reasons, higher osmolarity was used in this study to better mimic dry eye disease. In fact, the cells were cultured 370 mOsm/kg osmolarity (the lowest level of hyperosmolarity other than control medium) gave similar viability values to the control conditions (328 mOsm/kg) which is believed that 370 mOsm/kg culture conditions was not enough to induce the hyperosmolar stress in vitro. The hyperosmolar level used in this thesis was based on the study by Li et al., [226]. There is no internationally agreed consensus about the optimal levels of osmolarity to be used in vitro to mimic dry eye disease. Hence, different osmolarity was chosen to mimic the dry eye condition as; 370, 412 and 480 mOsm/kg.

There were two experimental designs in this study in which the light exposure was investigated; preoperative and postoperative dry eye. Assessment of a preoperative dry eye set up had more emphasis on light exposure since the post-operative results did not show any significant difference in their viability with light exposure (Figure 3 26).

The preoperative dry eye model, on the other hand, was shown to be adversely damaged with light exposure even though the only significant difference was observed for the 480 mOsm/kg culture conditions. There was a tendency of reduction in cell viability with light exposure within the same culture conditions even though the difference was not significant. This also indicates that the severe

dry eye condition (in this case 480 mOsm/kg) can be more prone to be affected by light exposure from the operating microscope. These results are in the line with clinical findings of worsening dry eye symptoms in preoperative dry eye patients who have undergone cataract surgery.

Light exposure was also found to affect the wound healing behaviour of the cells within the preoperative dry eye model. The wound healing was reduced particularly with higher hyperosmolar culture conditions; 412 mOsm/kg and 480 mOsm/kg culture conditions. Delayed wound healing is due to the effect of light exposure on the combination of cell proliferation and cell migration. The exact mechanism, however, needs to be confirmed with further studies to identify which pathway is more affected with the light exposure; either cell proliferation or migration. Delayed wound healing can also be a sign of loss of metabolic activity, cytoskeletal structures associated with cellular adhesion and apoptosis [235,273] which was also confirmed with Annexin V staining Figure 3-24.

The Live/Dead assay was able to highlight the wound area in different samples, but also indicated the status of cell health as live or dead. However, due to the lack of less magnification to image the whole well, only representative images in terms of wound area were chosen since the viability was confirmed and tested with the different assay. Therefore, live cell to dead cell ratio might not be very representative in Figure 3-17 since it is expected that dead cells would be found near a scratch, but not all the samples show this. The postoperative dry eye model did not show any significant cell viability damage with or without light exposure at 24-hour (Figure 3 26) and 48-hour culture time points (Figure 3-27). In fact, the cells completely recovered by the 48-hour time point. It can be concluded that light exposure caused more damage within already stressed cells. For this reason, it is believed that hyperosmolar stress after light exposure did not affect the viability of the cells.

These results confirm the hyperosmolar culture effect on conjunctival cells. These two experimental models confirm that any damage occurred in the preoperative dry eye can potentially have a greater impact in adverse environmental conditions such as light exposure during cataract surgery compared to non-dry eye (control) conditions.

For a better understanding of the light effect and cellular defence responses, cell size was also assessed. However, no difference between the different conditions was found either with light exposure or increasing hyperosmolarity. It is believed that this is due to the aggregation of the cells during analysis since instrument (Nucleocounter) was not able to remove the cell aggregation during the cell diameter analysis. It was expected that there would be an alteration in cell size difference, due to the hyperosmolarity in the cell medium. Cell size results displayed a tendency towards the enlargement of the cell diameter with increasing osmolarity which is believed to be due to regulatory volume increase. Higher extracellular osmolarity, like hyperosmolar culture medium, is known to cause greater loss of water and reduce the cell volume initially within 90-180 minutes [272] to achieve the balance of osmolarity between the extracellular and intracellular environments. After the initial shock, the adaptation process took place (termed regulatory volume increase) which recovers the volume of the cells [272,274,275]. Therefore, the tendency for the cell diameter to rise with increasing osmolarity could be explained due to the regulatory volume increase which is a defence mechanism of cells against hyperosmolar stress. [241]. Although cell diameter was found to have a tendency to increase with increasing hyperosmolarity, this was not statistically significant. The reason for not being able to detect any difference could be due to the culture time used in this study. Kiehl et al. [272] have shown that an increase in cell diameter within a hyperosmolar medium occurs after more than 96 hours. However, in this study, the time frame chosen to observe cell diameter changes might not be enough to see the difference since cell size changes have been observed under longer-term culture conditions for both preoperative and postoperative conditions [272].

In contrast to cell diameter, light exposure has been found to cause significant changes in cell apoptosis which is a programmed cell death [276]. When it is stimulated from external stimuli or internally, apoptotic cells display morphological changes such as cell shrinkage, membrane blebbing, cell detachment, externalisation of phosphatidylserine, nuclear condensation and fragmentation of DNA [277–280]. Necrosis, on the other hand, is acute cellular stress and can be caused by injury infection, stress, and it leads to loss of membrane integrity and the subsequent release of intracellular molecules to the outside [281–283]. Cell viability assays are not able to detect these cell death procedures since

MTT or PrestoBlue depends on mitochondrial electron transport chain and mitochondrial enzymes in cells which are both viable and metabolically active [281,282]. Reduction in cell viability on those assay indicates less metabolic activity and impairment, however it does not give any information about the mode of cell death or the pathway [281]. Therefore, this study also investigated the apoptosis rate to give more information beyond cell death.

It was shown that with higher hyperosmolarity and light exposure, cells were observed to be more apoptotic rather than following a necrotic by their higher prevalence in Annexin V staining rather than PI staining. The necrotic cell population distribution did not show a significant difference between the groups. Furthermore, the percentage of each condition in necrosis was quite small; between 5-15 % of the total Annexin V/PI stained cells.

Therefore, it can be concluded that the phototoxicity process will be affecting the cells with the prevailing role of apoptosis as Marek and her colleagues confirmed [168]. In apoptotic cells, the pre-apoptotic and late apoptotic cells were also analysed. From the apoptotic cells, most cells were predominantly found to be in the pre-apoptotic state rather than in late apoptosis, which can be reversible. The higher percentage of the cell population was found within the same culture conditions but with light exposed cells cultured in 412 mOsm/kg and 480 mOsm/kg, however, the difference was not significant. On the contrary, with 412 mOsm/kg, the most dramatic increase was observed in light-exposed samples compared to dark samples, confirming that light exposure caused more damage within the hyperosmolar medium.

Observations belonging to less osmolar media; control and 370 mOsm/kg conditions, throughout did not show much difference and behaved similarly, not only in Annexin V/PI staining but also for cell viability, wound healing and IL-6 levels. It is believed that 370 mOsm/kg hyperosmolarity was not enough to mimic hyperosmolar stress on conjunctival fibroblasts, which was also confirmed with other previous in vitro studies [213,226]. This result seems to be consistent with the other research by Li and his colleagues, which found similar behaviour in 370 mOsm medium conditions in terms of inflammation markers such as MMP-9 [226]. In fact, since they did not find any difference between

their control and 370 mOsm/kg medium conditions, they mostly focused on 400 -500 mOsm culture conditions to mimic hyperosmolar medium [226].

Although the light-exposed and dark samples at 480 mOsm/kg had the highest pre-apoptotic cell population, the difference between light-exposed and dark condition was very small. This was expected since the 480 mOsm/kg culture was already found to be highly detrimental, in which most of the cells were already damaged, was also confirmed with IL-6 levels and cell viability.

The pre-apoptotic state also indicates the possibility that recovery of the cells can be also considered as an additional pathway since the pre-apoptotic pathway is still reversible. In fact, Veronika et al. found recovery of apoptotic conjunctival epithelial cells after blue light exposure, however not all of the apoptotic cells were recovered fully [168]. This can also be interpreted along the same line as some of the findings of clinical studies. Dry eye symptoms in terms of conjunctival staining score after cataract surgery was found to be able to return to baseline after 1 month [284] or improved at three months postoperatively [102] which confirmed that our model gave results that support previous clinical findings. Longer culture of the preoperative model will be beneficial to investigate the recovery of the cells after light exposure. Therefore, future studies discriminating those two pathways with light exposure are required with fluorescently conjugated antibodies which specifically bind to intercellular apoptotic markers [279].

Inflammation has previously been shown to be proportional to hyperosmolar stress exposed to the ocular surface cells [215,226,285]. IL-6 has been receiving attention as a pro-inflammatory cytokine studied both in tear samples to investigate inflammation level and in vitro when studying both hyperosmolar stress and light exposure (visible and UV) [168,286]. Nevertheless, no study has explored the operating microscope influence on IL-6 levels to detect the inflammation afterwards.

In this study, the media of each sample was analysed for the secretion of IL-6 that had been released by the cultured cells, at the 24-hour time point after the light exposure. IL-6 levels were found to increase with light exposure and increasing osmolarity within control, 370 mOsm/kg and 412 mOsm/kg culture conditions. The higher increase with light exposure was obtained in 370 mOsm/kg and 412

mOsm/kg culture conditions compared to their dark samples. The light exposure from the operating microscope, which is predominantly blue light, led to rising inflammation in cells cultured within hyperosmolar stress. These results are in accordance with a recent study which examined the effect of blue light exposure on corneal and conjunctival epithelial cells when they were cultured in hyperosmolar medium. Although they exposed their cells to hyperosmolar stress for 7 hours before the light exposure, and light exposure duration was 17 hours long with irradiance with 1 to 1.5 Mw/cm². They found increasing IL-6 levels with light exposure when the conjunctival cell line was cultured in a hyperosmolar medium even though the irradiance they used was much less than our study light source (12.1 mW/cm²).

On the other hand, when fibroblasts cultured within a 480 mOsm/kg medium, the light-exposed samples did not show any difference in terms of their IL-6 levels in their medium compared to cells cultured in the dark. This finding is consistent with the previous observations made by Igarashi and his colleagues in which they found an increasing pattern with increasing IL-6 levels up to 700 mOsm/kg medium condition in vitro[215]. After that point, gradually decreasing of IL-6 levels were found with 800, 900 and 1000 mOsm/kg culture conditions in a dose-dependent manner [215]. Although they used higher osmolar culture medium conditions compared to our study, the results were still able to show consistency with our results found with the 480 mOsm/kg medium for longer-term culture. It is suggested that with this condition, fibroblasts were under too much stress so that they might have another activated pathway to downregulate the IL-6 production which should be further investigated with different inflammatory markers. Standard deviations are found to be quite large in Figure 3-25 as most of the other an analysis containing hyperosmolar stress. It is believed that this is mostly due to the hyperosmolar medium causing fluctuations in vitro which has been previously stated in the literature [168].

In conclusion, these results together further support the hypothesis that hyperosmolar stress can make the conjunctival fibroblasts more susceptible to light exposure from an operating microscope during cataract surgery. This chapter suggests that light exposure is a contributing factor for postoperative dry eye seen after cataract surgery. In addition, patients with preoperative dry eye can be affected

adversely by this same mechanism to a much greater extent compared to non-dry eye patients. This is the first study to explore the actual operating microscope's effect on conjunctival cells in an in vitro model. Moreover, this research carries significance for not only cataract surgery but any kind of ocular surgery in which a patient's eye is likely to undergo light exposure from an operating microscope, even those fitted with UV filters. Surgeons should take precautions against the exposure time of the ocular surface by the operating microscope illumination and also conditions of dry eye present before surgery. Patients with preoperative dry eye should be treated, for instance using appropriate therapies e.g the use of tear supplements before the surgery, to help improving the outcome. [176]**.**

In order to better understand the biological processes of how light exposure affects the cells, molecular studies such as gene expression would be useful to understand the effect at the gene level. Analysing reactive oxygen species, different inflammation markers such as MMP-9, TNF-α, IL-1β, IL-8 and TGF-β which have been shown to be involved in dry eye pathogenesis and wound healing and fibroblasts migration/proliferation should be considered. Observing mRNA expression of those markers should be useful to confirm the results of the present study and to provide more detailed information about inflammation and the ocular response against the light exposure. Longer culture time after light exposure could be also useful to assess if the cells are able to recover or not, which could be analysed using cell viability and inflammation analysis.

Although cataract surgery is usually relatively short compared to other types of ocular surgery, steps might need to be taken to minimise light exposure during surgery by reducing the light levels, minimising the duration of exposure and/or by using inbuilt safety filters within the operating microscope. Better clinical outcomes for patients means fewer complications. Patients can return to their daily routine much faster with an uncomplicated outcome. In the long term, increasing patient's satisfaction postoperatively will lead to fewer follow-ups and drug-prescription which will benefit to the health care providers in terms of cost and time eventually.

CHAPTER 4: THE UV IRRADIATION ON CONJUNCTIVAL CELLS

4 UV IRRADIATION ON CONJUNCTIVAL CELLS

OVERVIEW

The impact of ultraviolet (UV) irradiation on the retina has been extensively studied, however, very little attention has been paid to the role of ocular surface effect by the exposure of UV. It is known that UV irradiation is an important risk factor for ocular disorders such as pterygia [287]. Despite the importance of UV radiation on the ocular surface, there remains a paucity of evidence on the relationship between dry eye disease and UV exposure. The aim of this study was to explore the effect of UV radiation on conjunctival fibroblasts, with and without hyperosmolar stress to observe whether the hyperosmolar stress makes the cells more susceptible to UV radiation. Different wavelengths band cut off (340-390 nm) were used for 10 minutes on the conjunctival fibroblasts with different osmolarity (control, 370 and 412 mOsM/kg). The viability and the cell diameter was assessed after the exposure.

4.1 Introduction

The atmosphere plays a vital role in absorbing almost all of the UV-C (100-280 nm) as much as 99% [178]. UV-B (290–320 nm) and UV-A radiation (320–400 nm) are able to reach the Earth's surface, 10% of solar radiation of UV-B and 90-99% of solar radiation of UV-A are transmitted to the Earth [288,289]. Due to the increase in ozone depletion in the atmosphere, it is known that the level of UV-A, UV-B and UV-C radiation reaching to Earth's surface has increased, causing more detrimental effects on the human body [290]. A considerable amount of literature has been published on the detrimental effects of UV irradiation such as altered immune functions [291], skin cancer [292], age-related macular degeneration [293], cataract [294] and pterygium [295]. Owing to the higher energy level, UV-B radiation is responsible for most of the damage to health even though only 1% of the total UV radiation is UV-B [296]. However, UV-A damage on health has also been shown to induce melanoma, skin cancer by increasing pro-inflammatory cytokines, growth factors, generation of oxidative stress, extracellular matrix degradation and inflammation [289,297–302].

Apart from the skin, the ocular surface is the first barrier that UV directly reaches from the environment. Therefore, the ocular surface has a crucial role to protect the whole visual system from UV exposure. Prior studies have shown the effect of UV predominantly on the cornea [223,303,304], however, despite the importance, there remains a paucity of evidence about UV exposure on the conjunctiva which is known to be sensitive to UV damage [183]. Most of the existing research investigating UV exposure and conjunctival behaviour is related to pterygium which is a common ocular surface disease characterised by overgrowth of fibrovascular conjunctiva over the sclera then onto the cornea [189]. The pathology was shown to induce chronic inflammation, excessive cellular proliferation, connective tissue remodelling and neovascularization, which can eventually lead to vision loss [233,305].

On the other hand, little attention has been given to UV as a trigger for a dry eye [116,184]. However, there is evidence suggesting that UV is also considered to cause tear film instability and is capable of inducing dry eye [185]. However, there is no consensus about this relationship and there are

contradictory reports for claiming UV as the environmental risk factor for dry eye [186,306,307]. Furthermore, Hwang and his colleagues hypothesized that UV-B emitted by the operating microscope during surgery can cause instability of tear film, and so dry eye [116]. It has been speculated for several years that light of an operating microscope used during cataract surgery lead to toxic effects such as dry eye disease [116,169,170].

The UV exposure is also considered to worsen pre-existing dry eye conditions. It has been known that the tear film is capable of absorbing UV due to its antioxidants, therefore, it functions as a UV barrier [179]. A study was carried out by Choy et al. [179] with 120 subjects to relate the antioxidant capacity of the tear film and UV absorbing properties. They found that elderly patients who have reduced tear production, could be potentially more prone to corneal damage as the tear film's capacity to absorb UV light was reduced by dry eye disease. In addition, Hwang and his colleagues found increasing ozone levels were associated with dry eye disease in a Korean population with 16,824 participants [308]. Decreasing the quantity of tear production and also alterations in tear quality in dry eye disease reduces it's antioxidant levels, anti-inflammatory features and decreases its efficiency in absorbing UV [179]. Hence, any dysfunction of the tear film can make the cornea and conjunctiva prone to exposure to UV radiation directly in dry eye disease and potentially worsen the condition.

A recent in vitro study found that conjunctival epithelial cells under hyperosmolar stress did not recover from the UV radiation (390 nm) instead, corneal epithelial cells managed to return to the healthy state [168]. This can also be interpreted that dry eye conditions in vitro can be worsened by UV radiation. Moreover, conjunctiva has been found to be more susceptible to phototoxicity than the cornea. This trend was already demonstrated in terms of the strength of inflammatory response where the conjunctiva gave a considerably higher response than the cornea [168,263]. This is first of all due to the cornea's immune privileged feature. Secondly, the conjunctiva is highly vascularized and is contains immunocompetent cells, therefore, ocular surface inflammation initially begins with the conjunctival cells. As a result, the conjunctival cells are more responsive than the cornea to this stress [43,263,309].

The existing literature on UV radiation on ocular surface cells was also mostly focused on one or two filter bands of UV irradiation with limited wavelength differences. The limited published literature highlights the difference in wavelength in UV radiance effects between UV-B to UV-A [191,286,303,304], which is summarised in Table 4-2.

Author	Date	UV Wavelength and Magnitude	Cell Type	Main Findings
Girolamo et al.[286]	2002	UV-B, 40 mJ/cm2	Pterygium epithelial cells,	Il-6 and IL-6 were induced by UV-B radiation.
Girolamo et al. [310]	2003	UV-B 80 mJ/cm2	Human conjunctival and pterygium epithelial cells	Pterygium epithelial cells indicated more MMP-1 reactivity compared to conjunctival epithelial cells.
Schmut et al [192]	2003	UV-B from 312 nm, 30 mJ/cm2	Human conjunctival fibroblasts	Cell viability was reduced by 30%. with the UV-B irradiation. Iodine products were found to be beneficial for the prevention of the damage by UV-B in vitro.
Buron et al.[181]	2006	UV-C from 254nm, 30 J/m2	Conjunctival epithelial cell line (Chang cells (Wong-Kilbourne clone)	The cell death pathway was found with UV irradiation and Benzalkonium chloride. Caspase-dependent cell death was observed with UV irradiation.
Larrosa et al.[311]	2008	UV-B, 44, 66, 99 and 132 J/m2,	Human conjunctival epithelial cell lines	The hydro caffeic acid (HCAF) alone and as a mixture of HCAF with p-coumaric acid) was found to be effective to reduce UV-B induced cell damage in terms of viability and ROS generation
Viiri et al.[191]	2009	UV-B, 153 mJ/cm2	Human conjunctival epithelial and corneal cells	Urocanic acid was found to be useful to reduce IL-6, increase cell viability induced by UV-B irradiation
Chao et al.[190]	2013	UVA from 320 to 400 nm, 2, 5, 10, and 20 J/cm2	Human pterygial and conjunctival fibroblasts	Elevation of phosphorylated JNK, ERK was found with the UV-A irradiation
Abengózar-Vela et al.[312]	2015	UV-B, 200 mJ/cm2	Human corneal epithelial cells	UV-blocking contact lenses were tested to prevent the reduction in cell viability, increase in apoptosis and ROS production by UV-B exposure
Marek et al. [168]	2018	UV-B, 1.15 W/m² for 17 hour.	Human corneal and, conjunctival epithelial cell lines	Conjunctival cells were found to more prone to be UV and blue light phototoxicity than corneal cells.

Table 4-1. A summary of the literature investigating conjunctival cells exposed to UV irradiation.

Therefore, the present study was designed to investigate the effect of UV irradiance across a range of narrow UV bands on cellular viability and cell size under the hyperosmolar stress, which will give another insight to whether dry eye patients are more sensitive to UV radiation.

4.2 Materials and Methods

4.2.1 Cell Culture

Porcine conjunctival fibroblasts were obtained from porcine eyes taken freshly from a local abattoir. The comprehensive details of the procedures can be found in 2.2.1 Explant Dissection. Migrated fibroblasts were cultured from the explants in Dulbecco's modified Eagle's medium supplemented with 4% foetal bovine serum (FBS; F7524, Sigma-Aldrich, UK), 1% penicillin (10,000 units/ml) and streptomycin (10,000 µg/ml) 1% L-glutamine and 0.2 mg/ml Dextran. After reaching confluence on tissue culture plates, 10 mm petri dishes and 96 well plates were seeded with $5x\ 10^3$ cells/cm^2 seeding density. Cells belonged to p1 to p3 were used for all experiments in this chapter.

4.2.2 NaCl-Induced Hyperosmolar Stress

Hyperosmolar stress was created using additional NaCl to the already supplemented medium with 328 mOsm/kg (control) as described in section belongs to 2.4 NaCl-Induced Hyperosmolar Stress. Additional 30Mm, 50Mm, and 90Mm were added from 1M of NaCl to the supplemented medium, recorded as 370 mOsm/kg, 412 mOsm/kg and 470 mOsm/kg respectively, after the cells were cultured in petri dishes to reach 70 ± 10 % confluency. The media of the cells were changed every three days. The cells were cultured in hyperosmolar medium 48 hours prior to UV radiation.

4.2.3 UV Irradiation

The media of the petri dishes just covered the surface of petri dishes before the exposure. UV band filters with 340, 360, 370, 380 and 390 nm were used to expose the cells for 10 minutes with each medium condition. The controls were kept in the dark, compared to those exposed to UV. After the exposure, the media of the cells were renewed. The cells were kept in an incubator for 24 hours after

the exposure to assess the response of the cells. MTT results were repeated for four times. Other analyses were repeated for three times.

4.2.4 MTT Assay

3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT; Sigma-Aldrich, UK) is a colourimetric assay which is an accurate indicator of cellular viability [313]. The MTT assay was used according to the manufacturer's instructions, details can be found in 2.7 MTT Assay. Briefly, the fibroblasts were incubated with 0.5 mg/ml MTT for two hours. The MTT solution was then aspirated and the cells were washed with PBS. 100 µl dimethyl sulfoxide was added to each well for one hour. The plate was then read on a plate reader measuring absorbance with a wavelength of 570 nm.

4.2.5 Cell Count and Cell Diameter

Cell counts were performed with Nucleocounter 3000. The petri dishes, after 24 hours of UV exposure, were trypsinized with 0.25% Trypsin-EDTA (Lonza). Trypsin enzyme was inactivated after 5 minutes of incubation at 37°C with the supplemented culture medium. The solution was collected and centrifuged for about 5 minutes at 300 x g. The pellet was resuspended in the 5 ml of medium and 200 µl was taken to be counted with a Nucleocounter. Via-1 Cassettes were filled with the cell solution according to the manufacturer's protocol. The cell count, viability and average cell diameter was achieved with the help of viability assay with Via-1 Cassettes.

4.2.6 Statistical Approaches

Data were checked for normality with the skewness and kurtosis test to determine the appropriate parametric or non-parametric tests. In order to test for differences among groups for baseline characteristics and the various outcomes, independent t-test, one-way and two-way ANOVA were used.

4.3 Results

4.3.1 Spectra Results

The spectra of the UV filters were measured with the QE65000 spectrometer (Ocean Optics, Florida, US) between 245 and 390 nm. The spectrometer was calibrated against deuterium and tungsten-halogen standard lamps that had been calibrated by the (PTB), the national metrology institute of the Federal Republic of Germany. The spectra graphs of each wavelength band were indicated in Figure 4-1. The difference between the spectra graph with relative irradiance was indicated in Figure 4-2.

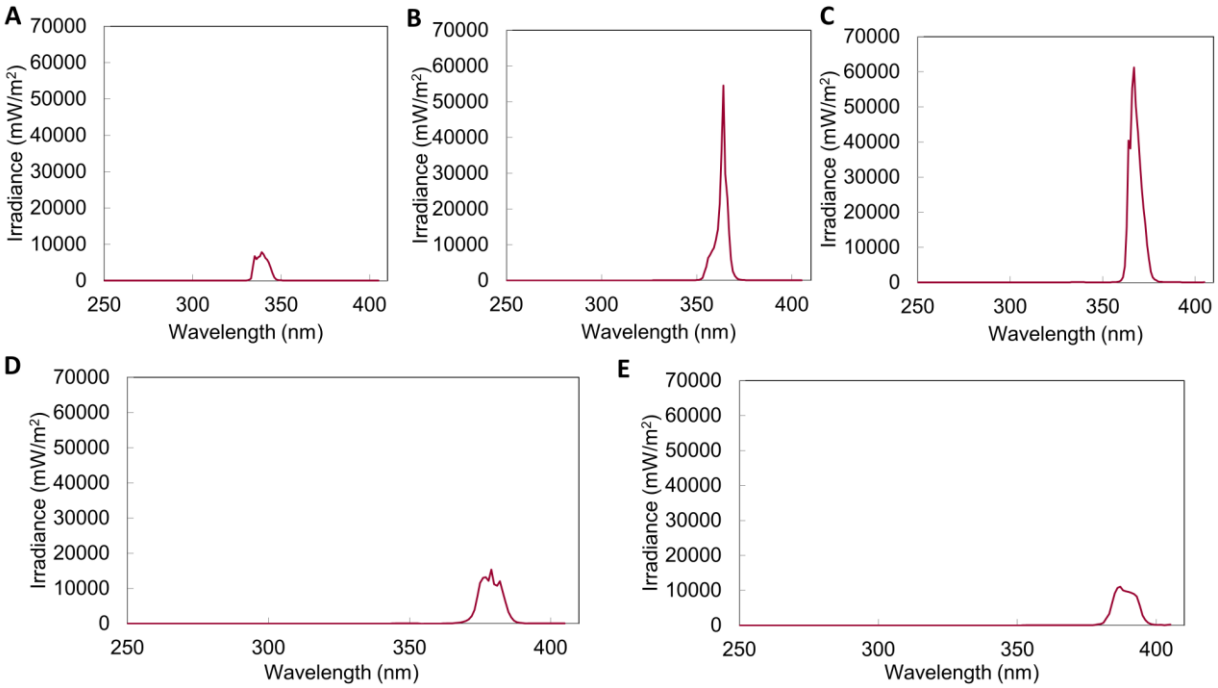

Figure 4-1. The spectra of the UV filters were shown. A. 340nm, B. 360nm, C. 370nm, D. 380nm, E.390nm UV filters spectra are shown. The sharpest spectra were achieved with the 370nm band.

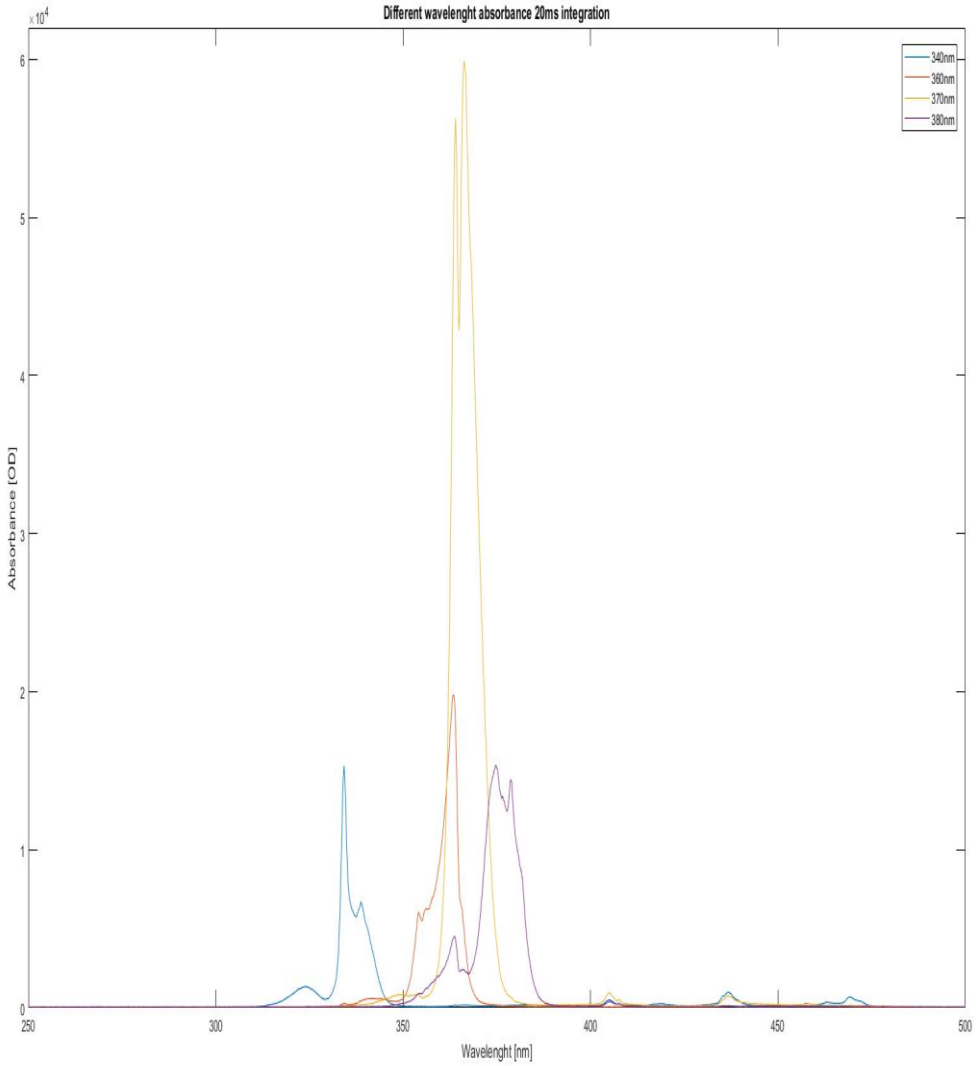

Figure 4-2. The spectral differences between UV filters are shown. Filter belongs to 390nm was not seen since the range of the spectrometer was not able to show in the same graph due to the small irradiance.

Irradiances of the UV filters are shown in Table 4-2. Total dose for 10 minutes of exposure was also calculated from the irradiance. The highest irradiation belongs to 370nm as 26.3 J/cm² followed by 15.3 J/cm² at 360nm, 8.4 J/cm² at 380nm, 6.72 J/cm² at 390nm and 4.3 J/cm² at the 340nm.

UV Filter Wavelength Band	Total UV irradiance (mW/cm²)	Total Dose for 10 minutes (J/cm²)
340nm	7.1	4.3
360nm	25.5	15.3
370nm	43.8	26.3
380nm	14	8.4
390nm	11.2	6.72

Table 4-2. Total irrigation and the energy of each UV filter band. The highest radiation/energy belongs to the 370nm band as expected due to the spectra data given above.

4.3.2 MTT Results

MTT was performed after 10 minutes of UV irradiation at the 24-hour time point. The results shown in Figure 4-3 are for the cell viability of conjunctival fibroblasts which are exposed to UV-A (340nm-390nm). Statistical significant was observed at the 340nm, 360nm and 370nm filter bands ($p<0.05$). The greatest reduction was found with the 370 nm filter, which caused almost 42 ± 8 % reduction in cell viability. Although having higher irradiation than the 340nm, UV from the 380nm and 390nm filter bands did not cause a significant reduction in cell viability.

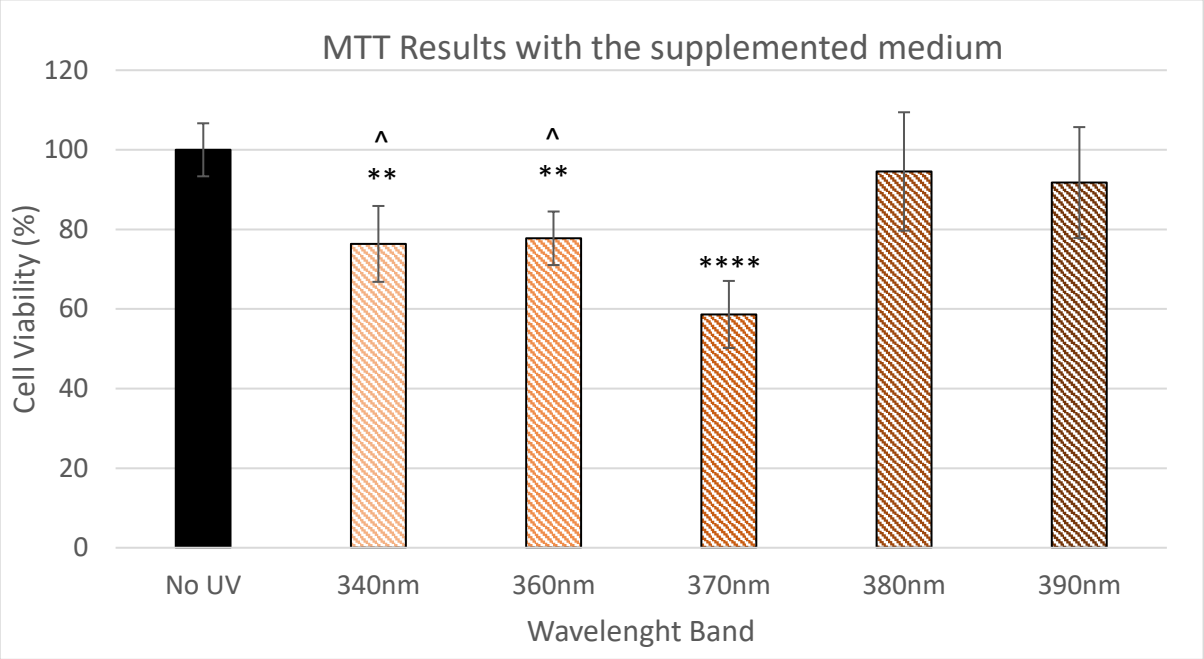

Figure 4-3. MTT results. The results for UV irradiation in the normal supplemented medium on conjunctival fibroblasts at 24-hours after 10 minutes of irradiation. Stars (*) represents the statistical significance compared to the dark values (no UV) (p<0.001). Carets (^) refer to the statistical significance compared to 370nm (*/^ p<0.05, ** p<0.01, **** p<0.0001).

The hyperosmolar medium was applied 48 hours before the UV radiation exposure. MTT assay was carried out after the 24-hour culture post-exposure of UV, shown in Figure 4 4. Hyperosmolar stress did not cause any difference in cell viability when there is no UV exposure. However, hyperosmolar stress with 412 mOsm/kg caused more reduction of cell viability generally within all the UV filters. On the contrary, the greater reduction was observed with the 370nm filter when the hyperosmolar stress was 412 mOsm/kg. With the 370 mOsm/kg hyperosmolar culture conditions, the viability was not affected for any conditions.

Figure 4-4. MTT results after UV exposure to hyperosmolar stress. The conjunctiva fibroblasts were treated with 10 minutes of irradiation with a variety of wavelengths. The MTT assay was performed at the 24-hour time point after treatment. A statistically significant reduction in cell viability was observed at 370nm UV at the highest hyperosmolar level (412 mOsm) compared to no UV conditions. Out of each UV band with three different culture conditions with different osmolarity level, only at 370nm UV band was found to cause a significant difference within different culture conditions. There is also a significant reduction when the cells were cultured in the 412 mOsm culture conditions and exposed to 340, 360 and 370 nm UV bands compared cells cultured in 412 mOsm but no UV conditions. Two-way ANOVA was used to detect the significance. Stars (*) refers to the statistical significance with the same hyperosmolar medium but no UV conditions. Carets (^) refers to the statistical significance of within different hyperosmolar medium but with the same UV exposure (*/^ p<0.05/^^ p<0.01, ***/^^^p<0.001).**

These viability differences do not show the differences between the filters since each filter has different irradiance. In order to see the differences on the cells with the weighted irradiance, the cell viability was normalised according to an irradiance of 360 nm, as shown in Figure 4-5. Although the 370 nm has the highest irradiance in 10 minutes, it was found that the 340 nm filter band affected cell viability the most if the irradiance of each band was equal.

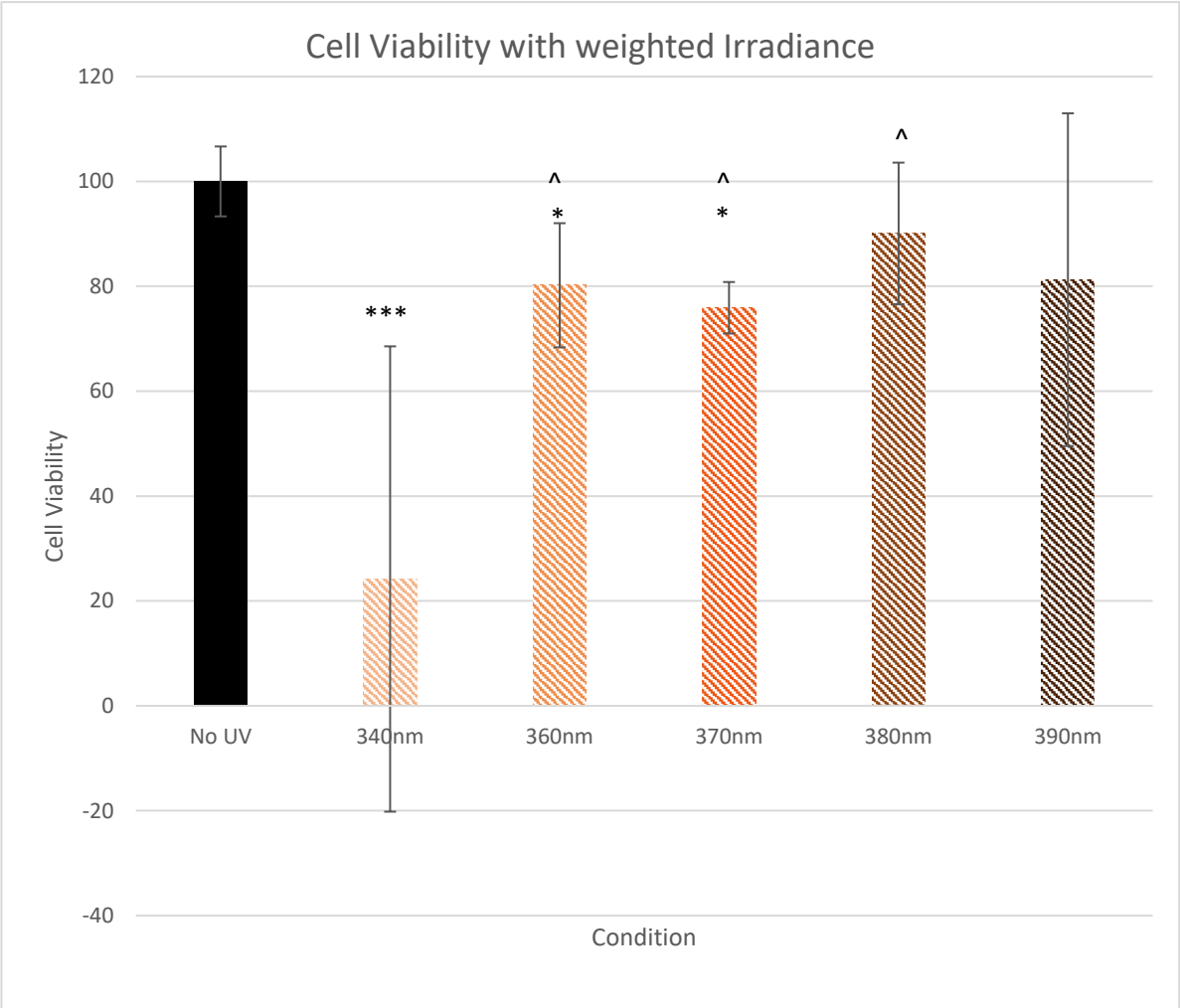

Figure 4-5. Cell viability with the weighted irradiance. Stars (*) refers to a significant decrease compared to no UV values. Carets (^) indicate the significant increase in cell viability compared to exposure to 340 nm filter. The most reduction was obtained with 340 nm filter band. (*/^ p<0.05, */^^^p<0.001).**

The hyperosmolar culture differences were also observed with the cell viability assessment. The control medium and 370 mOsm/kg did not show any difference for each of the exposure with different filters and no UV. With the 340nm filter, the cell reduction peaked. The 412 mOsm/kg culture medium caused more reduction in cell viability with all of the wavelength filters and no UV condition (except the 390 nm filter).

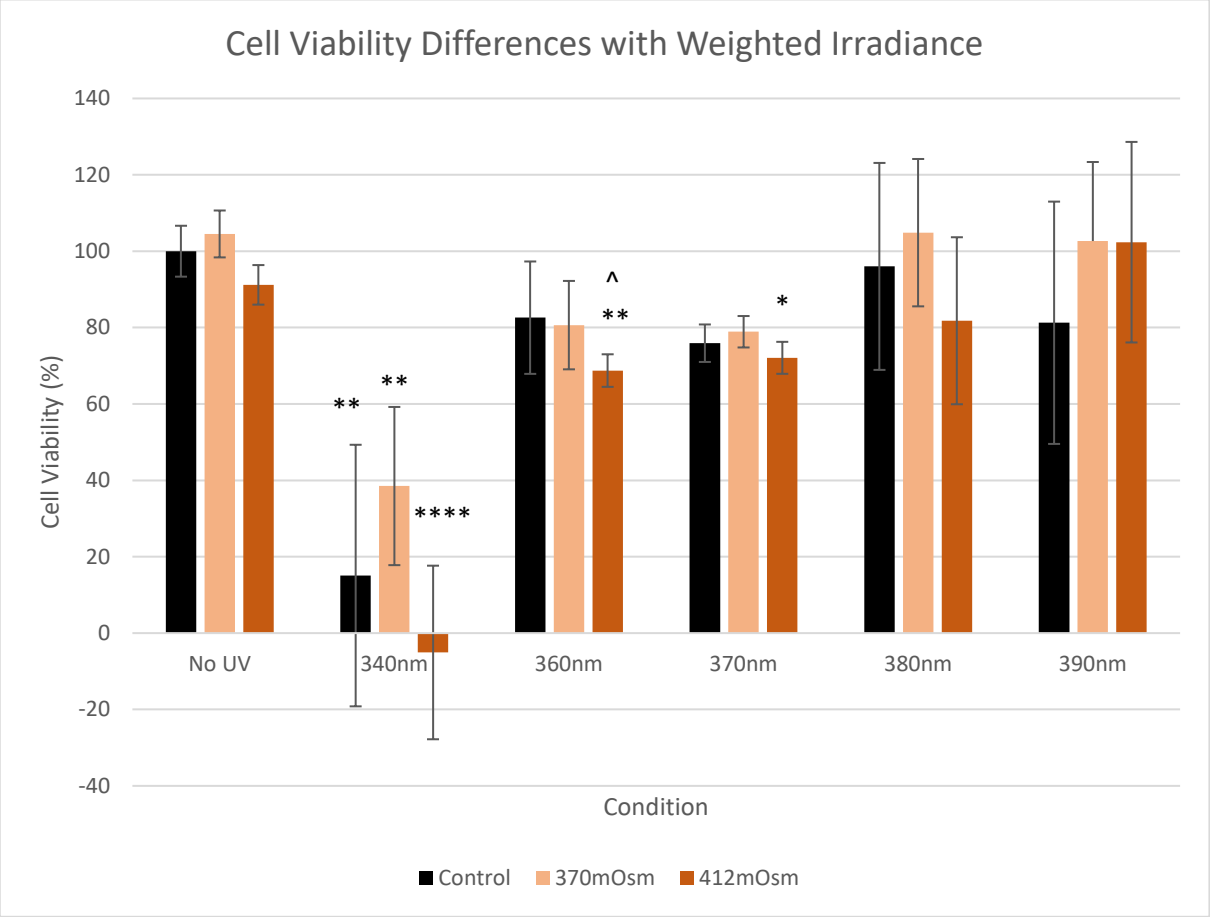

Figure 4-6. Cell viability differences with the weighted irradiance within different culture medium. Filter with 340nm almost killed all of the cells within 10 minutes of exposure. The 412 mOsm/kg culture conditions affected cell viability adversely. The greatest reduction of cell viability within each filter band group was observed within 412 mOsm/kg. Stars (*) refers to significant decrease compared to no UV condition within the same culture conditions. Carets (^) indicate the significant reduction in cell viability within the group. The greatest reduction was obtained with the 340 nm filter band. (*/^ p<0.05p<0.01, ****p<0.0001).**

4.3.3 Cell Diameter

Cell diameter with different stress conditions as determined by the Nucleocounter are shown in Figure 4-7 No statistical difference was observed within the groups, due to the aggregation of the cells during the counting. However, the reduction in cell diameter with the increasing radiation, especially for the 370nm filter, can be observed. The higher the osmolarity the smaller the diameter which is the case in most of the wavelength bands, except the 340nm. Decreasing cell diameter was not observed with the 380nm and 390nm filter bands.

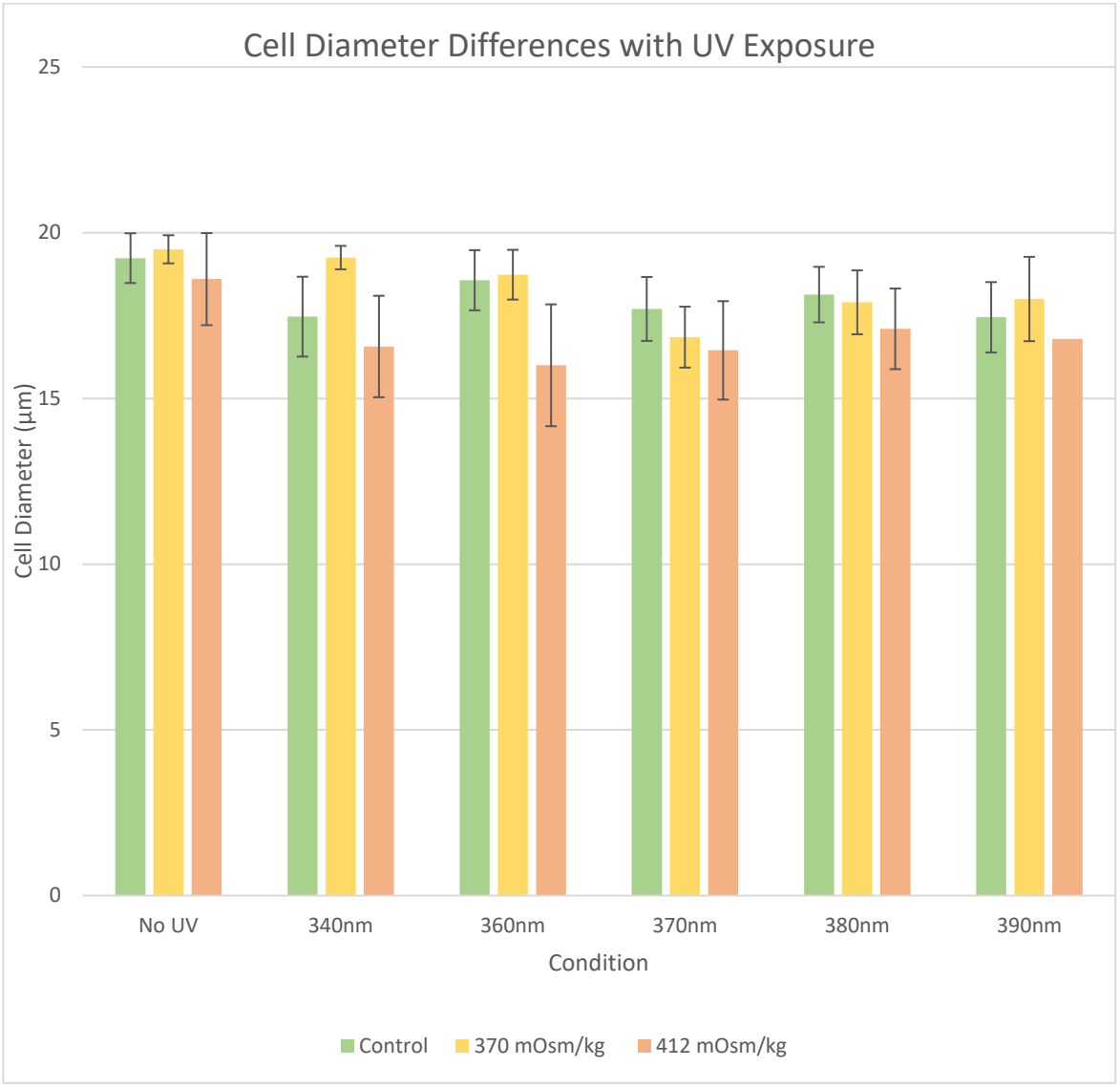

Figure 4-7 Cell size difference with different osmolarity and different wavelength irradiation. No statistically significant difference between any groups was observed ($p<0.05$) This might be due to the possible aggregation of the cells during the counting. Kruskal- Wallis and Friedman statistical test was used to detect possible differences.

4.4 Discussion

Environmental factors such as light have been known as a potential trigger to damage to the retina, and the ocular surface [256,295,314]. Whilst, a large body of literature regarding the effects of UV radiation on retina exists, the limited emphasis has been paid to the ocular surface so far despite the fact that all the external light radiation from the sun, light from the computer screens, laptops, smartphones, firstly arrive at the ocular surface. From those limited studies, most of the ocular surface studies analysing the effects on the ocular surface were predominantly about the cornea [304,315,316]. Most of the in vitro phototoxicity studies were based on the cornea. For instance,

corneal epithelial cell lines underwent apoptosis after UV-B radiation of 200 mJ/cm² for 5.5 minutes which is equivalent to solar UV-B radiation in July for an hour in Spain [312]**.** Even though a cell line was chosen to study the effect of UV-B, it still indicates the phototoxicity can have detrimental effect.

UV exposure on the eye was also explored as a cause of photokeratitis, cataract, pterygium and also corneal inflammation by causing elevation of pro-inflammatory markers [317,318]. It is known that UV irradiation is an important risk factor for ocular disorders such as pterygia [319] and conjunctival melanoma [293]. Hence, much of the current literature on phototoxicity with UV exposure on ocular surface pays attention to pterygium [190,320]. In fact, conjunctival fibroblasts were studied to understand pterygium due to excessive proliferation, extracellular breakdown and [190,264] UV-A irradiation of 1-10J/cm² from 90 seconds to 15 minutes [190]. Their MTT results after 24 hours indicated that the cell viability significantly reduced with 5 and 10 J/cm² but not with less irradiance [190]. In the current study, the viability results were not affected significantly with 6.72 and 8.4 j/cm² at 390nm and 380nm wavelength bands. However, above 8.4j/cm² cell viability was adversely influenced. On the contrary, 4.3 j/cm² gave a significant difference in the current study with the 340nm wavelength band which might be explained as the longer wavelength radiation is more tolerated by the cells compared to shorter wavelengths [321–323]. This was confirmed with the weighted irradiance of the UV light and re-calculated viability. Since the exposure time was constant, the scaling for the exposure level was possible as indicated in Figure 4-5. Cell viability with the weighted irradiance. Stars (*) refers to a significant decrease compared to no UV values. Carets (^) indicate the significant increase in cell viability compared to exposure to 340 nm filter. The most reduction was obtained with 340 nm filter band. (*/^ $p<0.05$, ***/^^^$p<0.001$). Figure 4-6. Cell viability differences with the weighted irradiance within different culture medium. Filter with 340nm almost killed all of the cells within 10 minutes of exposure. The 412 mOsm/kg culture conditions affected cell viability adversely. The greatest reduction of cell viability within each filter band group was observed within 412 mOsm/kg. Stars (*) refers to significant decrease compared to no UV condition within the same culture conditions. Carets (^) indicate the significant reduction in cell viability within the group. The greatest reduction was obtained with the 340 nm filter band. (*/^

p<0.05**p<0.01, ****p<0.0001). UV filter with 340nm was found to cause more damage with the weighted irradiance. This confirms that if the energy exposed was the same for all of the filter bands, the most cell damage will be observed with the 340 nm filter. Filters 360nm and 370nm will cause the most damage after 340nm. Cell damage was found to be minimum with the filter bands of 380 and 390 nm.

The tear film has a pivotal role on the ocular surface not only for keeping the ocular surface hydrated but also due to its UV-absorbing feature. In turn, the loss of homeostasis of the tear film is described as dry eye disease [60]. Evidence has demonstrated that antioxidant enzymes and tear production were reduced in dry eye disease which can impair the capacity of the tear film to absorb UV radiation [179,184]. This potentially predisposes to more serious damage to the ocular surface by UV radiation because the cornea and conjunctiva could be the first barrier to be exposed to UV light directly with insufficient UV absorbance from a dysfunctional tear film [179,324].

The results shown in Figure 4 3 suggest that UV-A light (with filters 340-390nm) can adversely affect cell viability in the control medium. As the exposure time was equal for each of the wavelength dependent filters, the energy differences can be seen clearly, especially at 370nm in Table 4 2. Cell viability was most affected in the samples cultured in hyperosmolarity medium used as a surrogate for a pre-operative dry eye seen Figure 4 4. There was not any significant change in the medium with the osmolarity 370mOsm/kg (compared to the control), perhaps due to the higher resistance to hyperosmolarity by the cells in vitro compared to in vivo (Clouzeau et al. 2012). This could explain why the viability results were not affected much with the 370mOsm medium. With 412 mOsm medium, the cell viability was more affected and the same trend was observed for each filter with the control medium.

Due to the high variation in the MTT results and also with the 450mOsm hyperosmolarity, this condition was not included in further cell diameter analysis. The results show high variation, however, the higher the osmolarity, the fewer viable cells were observed after UV irradiation. The lowest viability was observed at 370nm as expected, as the 370nm UV filter had the highest irradiance.

Cell size can be an indicator of apoptosis (Elmore 2007). The smaller the cell size, the more likely the cell is in an apoptotic state. Reduced cell size was observed with the highest hyperosmolar medium, 412mOsm and with the highest radiation at 370nm. No statistically significant differences between different conditions were observed. This is most likely because of the aggregation of the cells, inevitably occurred during the cell count even with using a vortex for mixing. Nucleocounter, the instrument used for analysing cell solution for cell diameter analysis was not able to assess the cell aggregation from the cell count. Even though there are no significant differences, the decreasing trend in cell diameter can still be observed with the increasing hyperosmolarity. With the 370nm wavelength band, the smallest diameter was achieved mostly due to to the highest radiation. Reduced cell diameter can be interpreted as a sign of early apoptosis since shrinkage of the cells, smaller size of cells and packed organelles have been observed during the early process of apoptosis [283,325].

In order to better understand and interpret the results of this study, the radiation level was compared to environmental UV exposure levels. In Paris, for example, UVA radiation from 11 AM to 1 PM in summer was measured as 54 W/m² and 19 J/cm² [190,326]. Ocular exposure was shown as 4.6 ± 1.9% for outside workers [326] and for carpenters 8.4 ± 1.4 %, this makes the total UV-A dosage between 0.6-1.3 J/cm² per hour for the outside workers, and for carpenters 1.4 to 2 J/cm². As a comparison, the UV-A levels used in this chapter vary between at least two hours being under the sun in summer in Paris (340nm dose, Table 4-2) 13 hours of exposure due to differences in radiation with wavelength bands. Although the irradiation from a 370nm filter which had the highest energy on the cells was probably more than a usual daily UV-A exposure on the ocular surface, the evidence presented in this section suggests the results indicate a trend towards reducing cell viability.

There is a growing body of literature of UV irrigation to ocular tissues in the last decades [168,304,327]. UV exposure from either environmental or from operating microscope during surgery can have undesirable consequences such as dry eye. Therefore, protection of the eye is important. There is still a lack of understanding in terms of safety because the level of UV radiation and also its

effect of an operating microscope on the ocular surface was investigated in a few studies only [116,169].

To protect the eye from environmental UV exposure, sunglasses, for instance, possess filtering classified from 0 to 4 in which 0 refers to luminance transmittance greater than 80% and 4 refers to the 3%-8% transmittance [328,329]. Unfortunately, protection from sunglasses is not enough to protect the eye as light is still able to reach to the eye depending on the solar angle and light orientation [330]. Whereas the significance of the UV radiation from the sun on skin has been well established, there is a lack of understanding of the effect of UV on the ocular surface and the role of various forms of protection [331].

As a summary, it is crucial to understand the effect of UV radiation on the ocular surface, to create better therapies for dry eye and conditions such as pterygium. The present study appears to be the first to combine both hyperosmolar stress and UV-R radiation in vitro conditions on conjunctival fibroblasts. Recently, Veronika et al., (2018) investigated the phototoxicity of blue light exposure with hyperosmolar stress [168] in which the same trend was observed by conjunctival and corneal epithelial cells as was also hypothesized in this study too. Therefore, it can be concluded that ocular surface cells under hyperosmolar stress are more sensitive to UV irradiation which is likely to worsen dry eye symptoms in vivo, although further clinical studies are required to confirm these results. This also implies that during cataract surgery, light from the operating microscope should be optimised to reduce exposure to potentially harmful levels of UV radiation by incorporating appropriate filters and/or minimising exposure times.

Furthermore, patients should also endeavour to protect their eyes from sun exposure after cataract surgery e.g using sunglasses. In addition, molecular studies such as differences in inflammatory cytokines, chemokines and reactive oxygen levels, are also required to explore possible associations between dry eye pathology and exposure to UV irradiation on a cellular level for better understanding of this phenomenon.

CHAPTER 5: POVIDONE IODINE EFFECT ON CONJUNCTIVAL CELLS

5 POVIDONE IODINE EFFECT ON CONJUNCTIVAL CELLS

Overview

Povidone Iodine (PVI) has been widely used in ophthalmic surgeries as an antiseptic agent. However, there has been a debate about the toxicity of this agent to the ocular surface. Moreover, there has not any consensus yet about the optimum concentration of the PVI. This study explored the effect of povidone iodine (PVI) on ocular surface to enhance the understanding of the dry eye pathology developed after cataract surgery since it has been considered as a potential factor for a postoperative dry eye. This study aims to observe the povidone iodine toxicity in terms of cell viability both in two-dimensional and three-dimensional in vitro model along with wound healing response on conjunctival fibroblasts. Different concentrations of PVI and different exposure time was tested to observe thecell viability responses.

5.1 Introduction

Health care professionals are constantly challenged by finding the best way of the prevention of infection by the microorganisms. Use of antiseptics before, during or after the surgical procedures is quite common. Povidone-iodine (PVI) is a frequently used antiseptic agent in laboratories, for the disinfection of patient's as well as health care provider's skin [332–334]. Moreover, it is commonly used for disinfection of the spine [335], the ocular surface [253] and also for treating the contaminated wounds due to a broad spectrum of antimicrobial activity and its capacity to speed up the wound healing even though there are contradictory reports regarding wound healing properties [118,229,336].

PVI use has been preferred in wound healing to decrease the use of antibiotics since resistance to antibiotics has been found to increase in recent years. Hence, as an alternative option for a microbicidal, PVI has been taking attention. PVI has been preferred owing to a broader range of antimicrobial activity, furthermore, it also prevents the possibility of developing resistance through their mechanism of action. Therefore, PVI use has been favoured to use instead of antibiotics [337,338].

Antimicrobial activity of PVI depends on the level of free iodine which is in the equilibrium with its carrier in polyvinylpyrrolidone (PVP) [339]. PVP is a synthetic carrier polymer and it has no microbicidal properties in itself. Free iodine is released from the PVI complex into the solution which is the crucial part of PVI efficacy. Therefore, microbial toxicity depends on the amount of free iodine in the solution which alters according to the concentration of PVI. When PVI releases the free iodine into the solution, the toxicity is initiated by free iodine attacking the cell membranes of the pathogen. Its mechanism of action is through the oxidation of the nucleotides fatty/amino acid, destabilisation of the structural elements of the cell membrane, which cause denaturation and deactivation of them. It is known that PVI oxidizes nucleic acids and cytosolic enzymes in the respiratory chain in bacteria and in the cell wall of fungi [339]. As a result, it leads to permanent damage to the pathogen within seconds [229,340].

No consensus exists about the optimum concentration of the PVI efficacy and toxicity to the host even though 5-10% of PVI is the generally chosen concentration. There have been interesting due to variation results from the studies which have explored the effect of the concentration of PVI used [114,197,341]. Diluted PVI solutions kill numerous viruses, bacteria, fungi and some other parasites and are thought to be safe and tolerable. PVI exposure has been shown to be effective against pathogenic bacteria such as *Staphylococcus aureus* and *Pseudomonas* spp in concentrations as low as 0.1 % [342,343]. Studies showed that higher concentration such as 10 % of PVI may actually be less effective in terms of bactericidal activity than lower concentration such as 1% of PVI [115,197,341]. Diluted PVI was found to release more free iodine into the solution even though measuring free iodine levels is still controversial due to the complexity of the chemical structure of the iodine [197,251].

The toxicity to the host tissue and delayed wound healing have been also reported along with exposure. Even though the exposure of PVI is well established on the skin in a number of in vitro and in vivo studies [332–334,344,345]. There is no consensus about its effect on the host tissue. A considerable amount of in vitro studies suggesting impaired wound healing effect [118,334,346]**,** no consistent deleterious effect of PVI has been observed in vivo as yet. Few of the in vivo studies found that PVI irrigation did not inhibit the granulation and epithelialization procedures during wound healing. [344,347]. Wang et al. recently found that exposure to 0.5 % PVI enhanced wound healing behaviour for an hour per day for the first 5 days in the dorsal skin of rats [348]. On the other hand, Thomas et al. observed delayed wound healing on dermal fibroblasts with 0.2% of PVI exposure for 24 hours [118].

Balin and Pratt [334] investigated the toxicity of PVI on the growth of human dermal fibroblasts by exposing cultures to PVI for prolonged times of 20, 24, 30, 48 and 72 hours. They found that 0.1% of PVI exposure and greater concentrations completely inhibited cell survival and exposure to 0.01% and 0.025% PVI delayed cell growth [334]. A recent study by Liu et al. [349] investigated the effect of povidone-iodine at different concentrations on wound healing response from fibroblasts, osteoblasts and myoblasts by using a scratch assay. The cells were exposed to 3 minutes of PVI treatment and found that the scratch defect stayed the same indefinitely with greater than 0.1 % concentration of

PVI even at 14 days after the scratch was created. It was suggested that PVI can potentially inhibit fibroblast proliferation and suppress the migration to close the wound [334]. Due to the differences in the experimental setup, inconsistent results were observed with PVI exposure. Most of the emphasis in the literature was based on skin and its response to PVI exposure despite the fact that it has been commonly used in ophthalmic procedures.

PVI has been used as an antiseptic agent in ophthalmological applications since the 1960s [350] and is currently the standard of care as a method of ocular surface preparation prior to cataract surgery to prevent endophthalmitis [351]. To reduce bacteria in the wound area, 5-10% of PVI is applied to the cornea, conjunctival sac and periocular skin for a minimum of three minutes prior to surgery as a standard routine recommended by European Society of Cataract and Refractive Surgery [351]. Possible cytotoxicity of PVI on the ocular surface has recently received attention since it has been also thought as one of the risk factors for dry eye after cataract surgery [128,352]. However, only a limited number of research has evaluated the association between PVI use and dry eye after cataract surgery clinically or in vitro. Ridder et al.,[352] found significant changes in visual acuity and an increase in subjective complaints with PVI exposure. The damage was observed with corneal staining with 2-3 minutes of 5% PVI exposure at 1-hour, 4-hour and 24-hour with 10 patients. Administration of topical anaesthetic was performed before the PVI exposure which could have also contributed to the corneal damage [353]. Therefore, it is not really clear whether the PVI exposure and/or anaesthetic agent causes the ocular surface changes, which is the limitation of this study. Jiang et al. [200] exposed rabbit eyes to 5 % PVI which caused significant changes in the corneal epithelium which was recorded as severely damaged using fluorescein staining. Additionally, rabbit eyes exposed to different PVI concentrations for 30 minutes. The indicated damage that was found to be concentration dependent; more damage was observed with increasing concentration of PVI from 0.5%, 1%, 2.5% and 5%. This study did not rinse the eyes after the PVI exposure which is the main limitation since the human eyes during cataract surgery are washed after a certain amount of exposure to PVI (mostly 3 minutes). Moreover, the exposure duration was longer than the 3 minutes which is the most commonly used for ocular surface antisepsis [115].

Yanai et al,[199] also found that barrier function of human corneal epithelial cells has been significantly damaged with 1.25% PVI exposure for 10 minutes even though the duration of exposure was longer than used in in vivo conditions [199]. Furthermore, the corneal stromal toxicity was also investigated with PVI exposure since when the epithelial layer is absent or damaged, corneal fibroblasts will also be exposed to PVI directly. Toxicity was found with 0.25 % PVI for 2 minutes in vitro with corneal fibroblasts [202]. Until now, no study has explored the conjunctival stromal irrigation with PVI in vitro.

The variation of the in vitro results reported is mostly due to the lack of validated in vitro models, using different cell types, and exposure times. In vitro models were also criticised for not being able to provide the same environment as cells in vivo within their matrix as the toxicity of PVI exposure results can show differences compared to in vivo. To overcome this disadvantage, three-dimensional in vitro models have evolved and are used commonly for a variety of purposes. PVI exposure was tested in skin three-dimensional models to observe the penetration of PVI and toxicity. PVI exposure was found to slow the wound healing and found to be toxic to fibroblasts [354]. However, to date, no ocular surface cells within the three-dimensional matrix have been studied with PVI exposure.

On the other hand, one of the reasons for discrepancies is that alterations could be also due to the choice of PVI since the properties can change with the different manufacturers since the minimum toxicity level has been differed in human cells in different in vitro studies [200]. Therefore, in this thesis there are two different PVI was used to observe possible differences between the two kinds. Nex Iodio (Nex Medical Antiseptics, Milan, Italy) was used for surgical washing of hands and the Minims (MIMIS, London, UK), was used for pre-ocular and conjunctival antisepsis prior to ocular surgery.

The literature suggests that at higher concentrations, PVI can cause conjunctival irritation [355], corneal epithelial damage [200], corneal oedema [356], damage to corneal fibroblasts [202], stinging [357]and allergy (contact dermatitis) [358]. One report suggests that if PVI is used at the end of cataract surgery (not current practice), it could lead to IOL opacification with certain silicone IOL materials [359]. It is unclear to what extent some of these ocular side effects of PVI could account for the dry eye symptoms often reported by patients following cataract surgery [199]. One of the reasons is that the scientific literature lacks details on the precise effect of PVI exposure on the conjunctiva. Even

though it is one of the key tissues involved in dry eye pathogenesis and ocular surface inflammation, conjunctival behaviour to PVI exposure needs to be better understood [128,352]. Therefore, this study aims to assess the conjunctival fibroblasts response to PVI exposure with different PVI concentration and different exposure times in vitro. Both monolayer, and three-dimensional in vitro models with fibroblasts embedded in collagen gel were visualized to observe possible toxicity effects. Wound healing was also monitored with exposure to different concentrations of PVI to investigate the potential wound healing response.

5.2 Materials and Methods

Porcine conjunctival fibroblasts were obtained by culturing conjunctiva pieces from freshly taken porcine eyes. A detailed description of the dissection and culturing procedures can be found in Chapter 2.

5.2.1 Two Dimensional Cell Culture

Porcine conjunctival fibroblasts were cultured from p1 up to p4 for these experiments. The control medium contained DMEM supplemented with 1% Penicillin/Streptomycin and 10% FBS, details of the cell culture procedure can be found in 2.2 Cell Culture. The cell density of $5x\ 10^3$ was kept constant for each experiment on the monolayer. For viability and wound healing assays, 96 well plates were seeded. Both assays were performed when the cells reached more than 70% confluency.

5.2.2 Three Dimensional Culture

Conjunctival fibroblasts were used when the flasks were ready to be passaged. Cell counting was performed to take 1×10^6 cells per 3 ml of collagen gel. Collagen gel was performed simultaneously with the cell count in order to prevent the initiation of gelation. All the components and collagen gel were all kept on ice to maintain the liquid form before mixing all the constituents. For every 3 ml of gel, 1,485µl of collagen gel (Corning, Amsterdam, Netherlands), 281µl of distilled water, 34.1µl of

NaOH (1M), and 200 µl of 10x DMEM (Sigma, Dorset, UK) were mixed in the bijous by keeping it on top of the ice.

1 x 10^6 cells were separated from the cell solution and centrifuged at 300 x g. The supernatant was removed, the last 100 µl of supernatant were left to resuspend the pellet in the tube. After mixing, 100 µl of the cell solution was mixed with the collagen gel. The collagen gel mixture and cell's solution were mixed until the colour of the solution was constant throughout. The 12 well plates were used to put the gels in. Polytetrafluoroethylene (PTFE) pieces were placed on each well of 12 well plates before the gels were put. 100 µl of gel solution was placed on top of the PTFE for each well and incubated for 2 hours at 37°C with 5% CO_2. Once hydrogels were set, the wells were topped up with the supplemented medium. The hydrogels medium was renewed every 3 days before the PVI exposure.

5.2.3 Povidone Iodine Dosing

Two different types of PVI were used to test on conjunctival fibroblasts. The Nex Iodio P2 PVP-I 5% ((Nex Medical Antiseptics, Milan, Italy) and comes in a ready to use 1000ml bottle as it is used as a scrub for surgical hand disinfection. Minims® Povidone Iodine 5% (MIMIS, London, UK) is used for ophthalmic application and comes in 0.4 ml containers for single use only.

Different PVI concentrations were prepared with dilution from 5% to 0.05% of PVI with the supplemented medium. (There was no difference in using PBS or saline for dilution instead of supplemented medium, therefore, supplemented medium was chosen). The media was discarded and the PVI solution was placed into the wells for the desired exposure time (30, 60, 120 seconds or 3 minutes). The PVI solution was then discarded and the wells washed with PBS immediately after. When the wells were terminated immediately, the assay solution was put into the wells instead of the culture medium after the washing step. For longer culture times, the supplemented medium was subsequently added back until the termination. For the control well, cells were cultured without any treatment but had regular medium changes like the other wells until the termination.

5.2.4 Wound Healing

A scratch was created manually just before the three minutes of exposure to PVI, details of the scratch process can be found in Chapter 2, 2.5 Wound Healing Assay. Different concentration of PVI exposure was put into the wells as described in the previous section after the scratch was created, (i.e. from 5 % to 0.05 %).

5.2.5 Viability Assays

5.2.5.1 *PrestoBlue Assay*

The supplemented medium was used to prepare 10% of PrestoBlue assay solution. Before the assay initiation, the media of the wells were discarded and 100 µl of presto blue assay solution was put to each well in the 96 well plates. The cells were cultured for 2 hours with the presto blue in the wells in the dark in an incubator at 37°C with 5% CO_2. After 2 hours of incubation, the fluorescence values were read using a 560nm excitation and 590nm emission filter with a fluorescence plate reader. Details of the procedure can be found in Chapter 2, the section of 2.7 MTT Assay and 2.8 Presto Blue Assay.

5.2.5.2 *Live/Dead Assay*

Live/Dead staining was used to visualise the effect of the PVI on both two dimensional and three-dimensional cultures at the 24-hour time points. This imaging technique was used for the concentration-dependent studies and for monitoring the effect of different concentration of PVI on wound healing. Live cells were distinguished by Calcein-AM with green fluorescence, whereas Ethidium homodimer (EthD-1) stained the dead cells with red fluorescence

5.2.6 Imaging

All of the imaging analysis after PVI exposure were performed at the 24-hour time point. A minimum of three samples per condition were performed, representative images were used in this section.

5.2.6.1 *Light Microscopy*

Imaging of scratch assay was carried out with a light microscope (Motic, Wetzlar, Germany) at a 24-hour time point before any assay was performed.

5.2.6.2 *Fluorescence Microscopy*

The images were taken using fluorescence microscopy. Details of how the assay was carried out, are described at Chapter 2.6 Live/Dead Assay. Alive cells were visualised with 495nm excitation and dead cells were visualised with 515nm emission.

5.3 Results

5.3.1 Cell Viability Results

Quantitative cell viability was measured with presto blue assay after PVI exposure at time 0, 24 hours and 96 hours. Fluorescence values from presto blue were normalised with reference to the mean control values (as 100 %).

The effect of 3 minutes of PVI exposure using Nex Iodio at time 0 and 24-hour is shown in Figure 5-1. Both time 0 and after 24-hour culture conditions in cell proliferation are indicated.

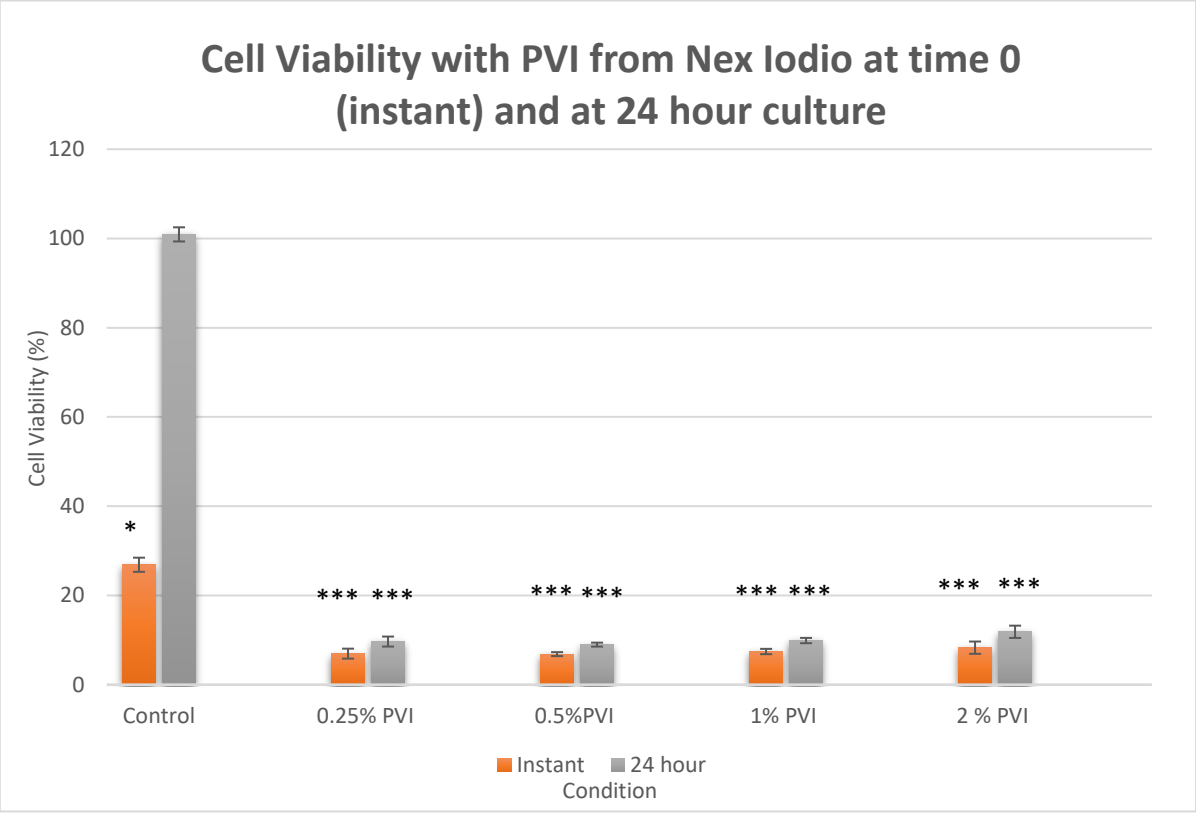

Figure 5-1. The presto blue assay shown at the time 0 and at 24 hours following 3 minutes of PVI treatment. The fluorescence values were normalised according to the mean of the control (no treatment) values. All of the PVI concentrations caused significantly reduced viability at both time points. Student t-test was applied (*p<0.001, *p<0.05).**

Due to the dramatic effect obtained at time 0, the rest of the results were designed to assess longer culture time (≥24hour) to observe whether the cells recover after the PVI exposure.

The effect of different PVI (Minims) exposure times from 30 seconds, 60 seconds and 120 seconds was observed at 24 hours and 96-hour time point. PVI concentration of 5%, 1% 0.5%, 0.1% and 0.05% were used. Cell viability at 24 hours was recorded for 30 seconds exposure of PVI in Figure 5-2, 60 seconds in Figure 5-3 and 120 seconds in Figure 5-4. The overall summary of all three graphs was represented in Figure 5-5 for 24 hours.

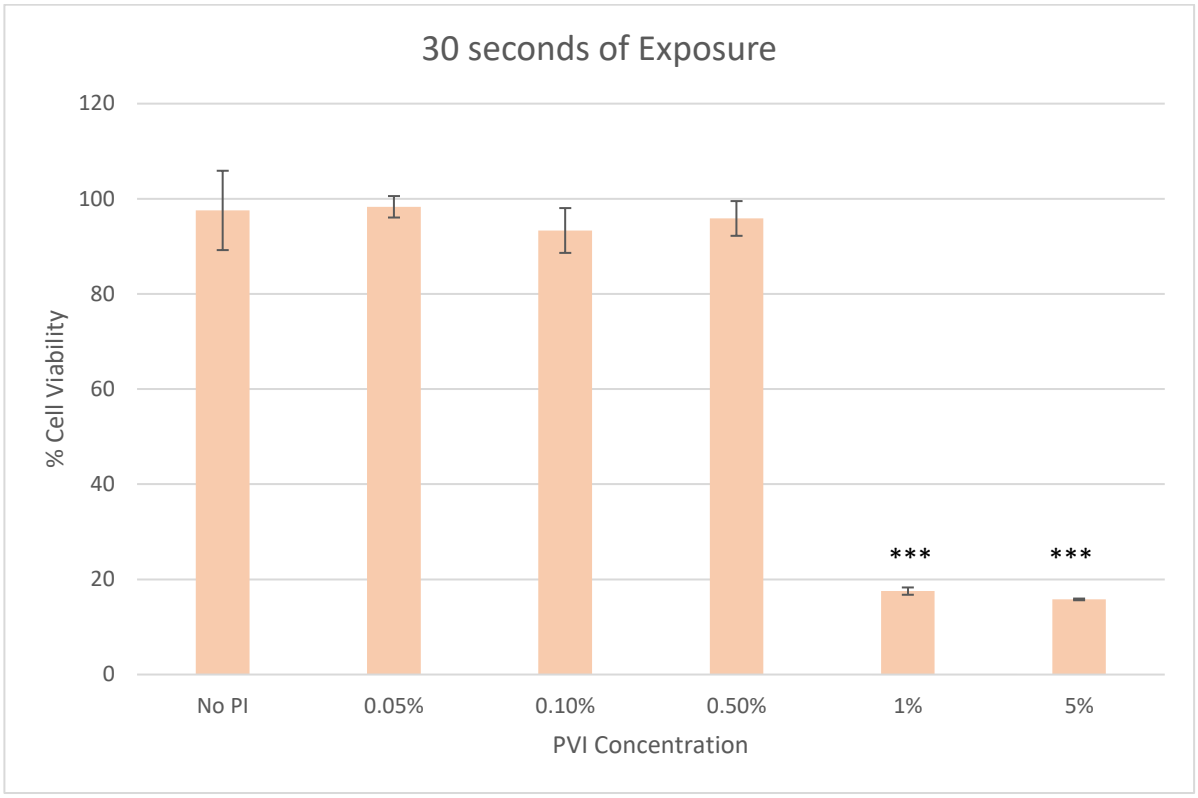

Figure 5-2. The cell viability assessed by presto blue at the 24-hour time point after exposure with a variety of different PVI concentration for 30 seconds. Significant reduction with 1% and 5% of PVI exposure were obtained with Student t-test. (*p<0.001)**

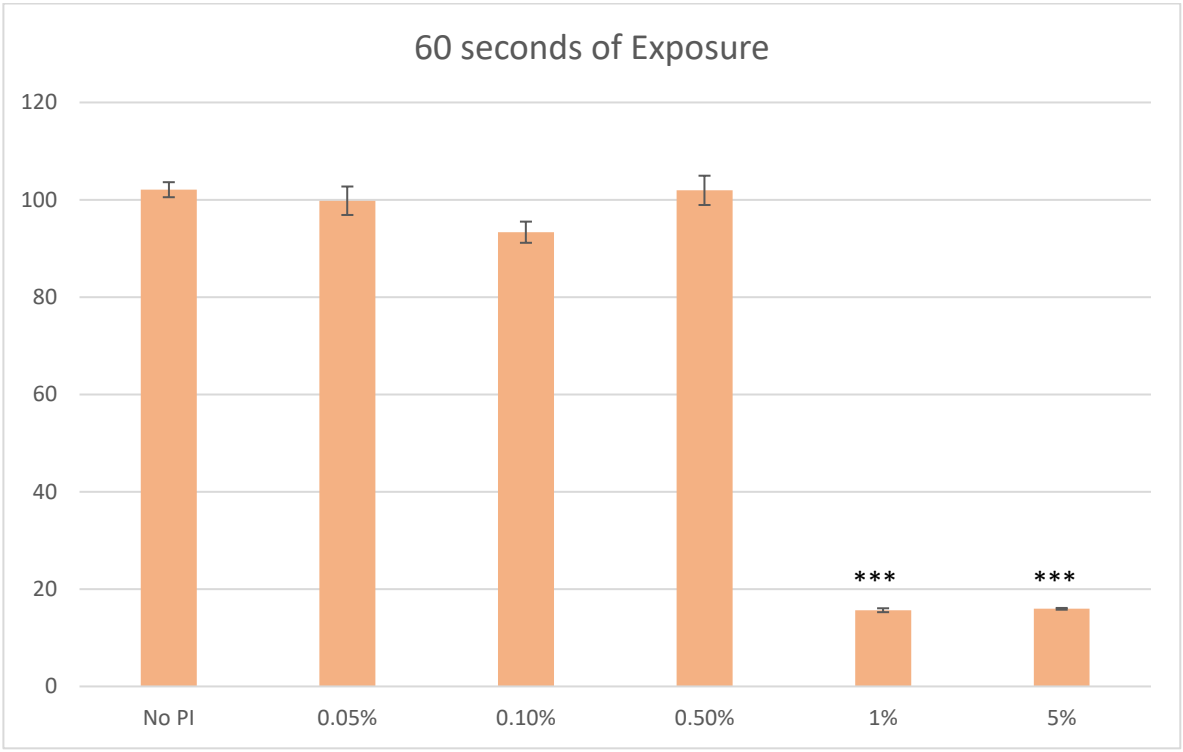

Figure 5-3. Cell viability at the 24-hour time point after 60 seconds of different concentration of PVI exposure. Student t-test performed to test for statistically significant reduction at 1% and 5% of PVI exposure. (*p<0.001)**

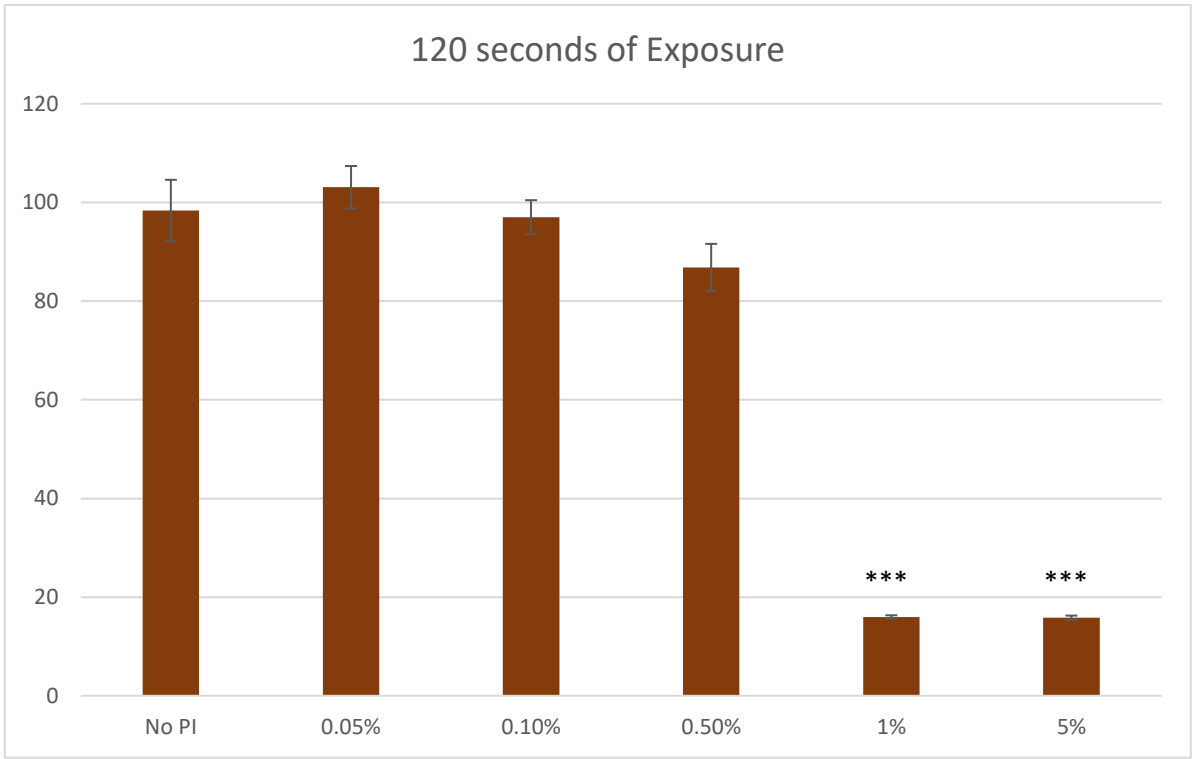

Figure 5-4. Cell viability difference with a variety of PVI concentrations for 120 seconds exposure at 24-hour time point. Significant reduction in cell viability was observed with 1% and 5% of PVI exposure. Student t-test performed (*p<0.001)**

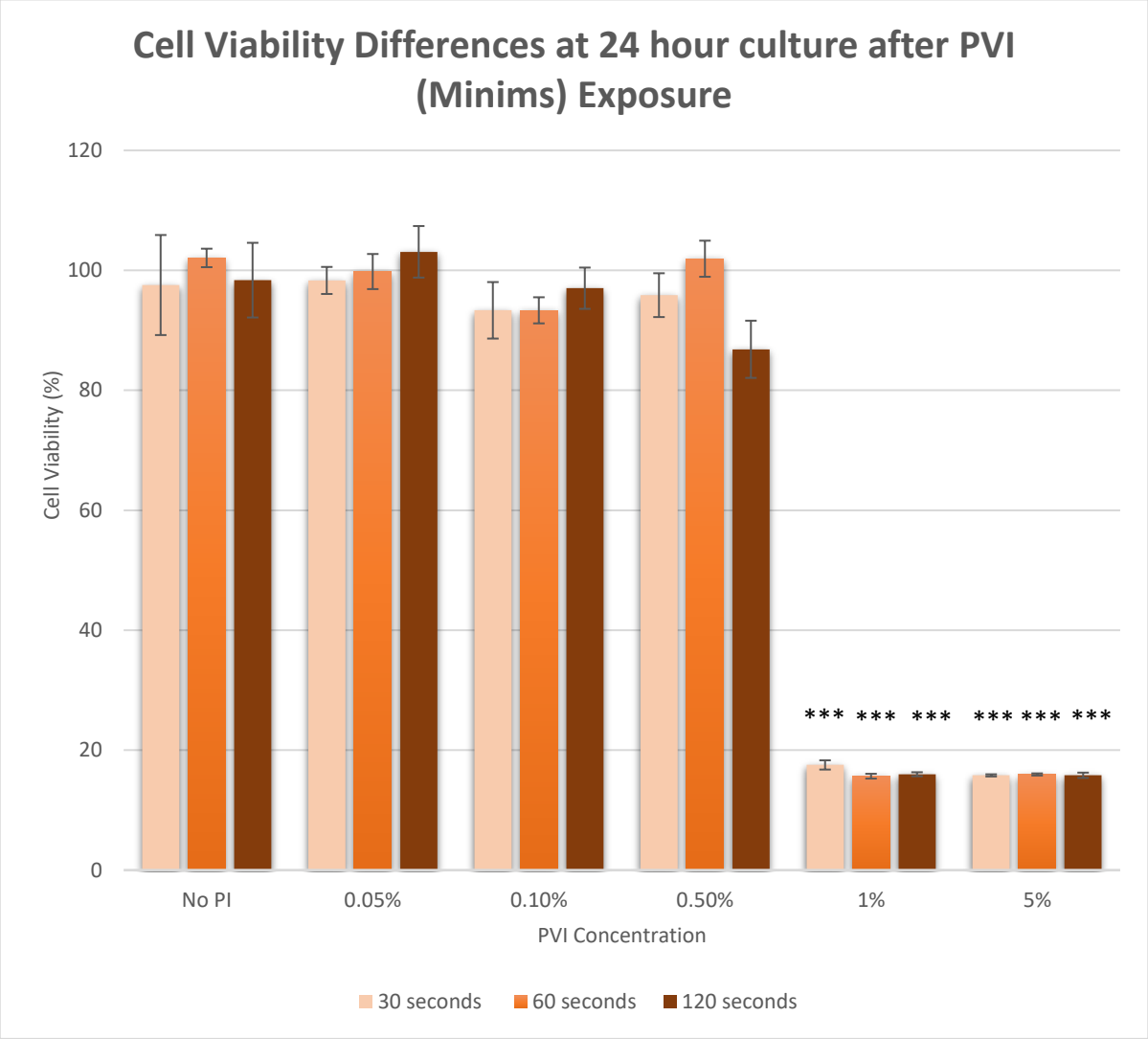

Figure 5-5. Summary of the PVI exposure of 30 seconds, 60 seconds and 120 seconds at 24-hour culture. Figure 5-2, Figure 5-3 and Figure 5-4 Error! Reference source not found. **was summarized. The viability of the cells was significantly decreased difference at 5 % of PVI and 1 % of PVI independently from the exposure duration. Stars represent the significant reduction compared to control (No PI) (****p<0.001).**

The effect PVI exposure on cells following longer culture (96 hours) can be seen in Figure 5-6. A small recovery of cell viability can only be observed at 1 % of PVI exposure when the exposure was 30 seconds however it did not recover completely. Cell viability did not recover at 5% of PVI exposure for 30 seconds, 60 seconds and 120 seconds, and at 1% of PVI, no recovery was observed with 60 seconds and 120 seconds exposure. At lower than 1% of PVI concentration, the viability was not affected by different durations of exposure.

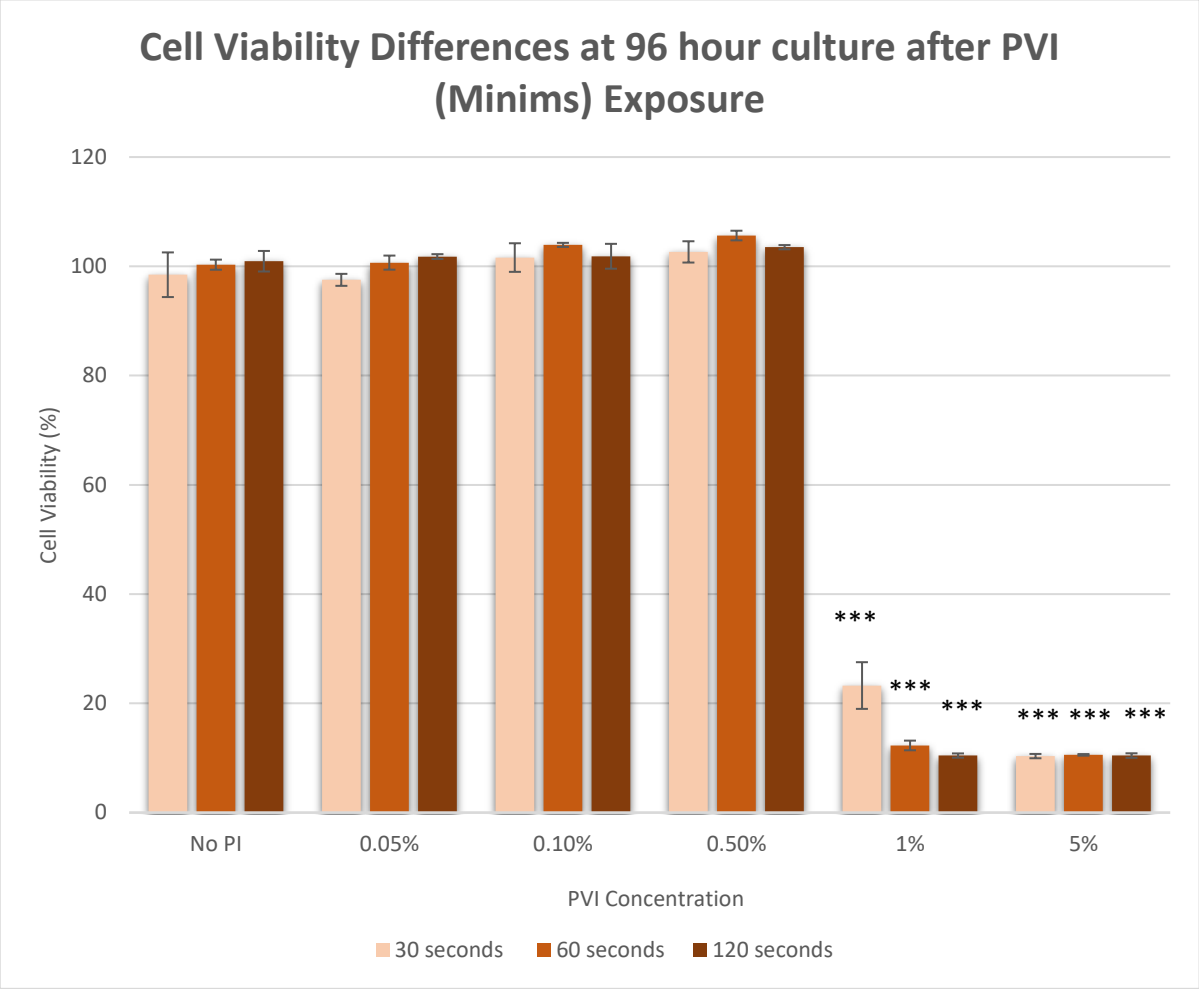

Figure 5-6. Cell viability for different concentration and after different culture times using the PrestoBlue assay shown. Differences detected at 5% and 1% concentrations, the viability of conjunctival fibroblasts was significantly reduced compared to other concentrations and no treatment. Recovery of cells was significantly higher in 30 seconds of exposure compared to 60 and 120 seconds at 1% of PVI (p=0.04). Less than 1% of the PVI concentration indicated almost no effect in cell viability with all durations. Student t-tests were performed. Stars represent a significant reduction compared to control (No PI) (**p<0.001).**

PVI exposure is generally applied for 3 minutes in routine surgical applications. Therefore, exposure to 3 minutes of PVI (Minims) was observed with cell viability. Cell viability was assessed at 24-hour and 96-hour time point to observe the recovery, seen in Figure 5-7 and Figure 5-8. The only recovery was seen for 0.5% of PVI exposure.

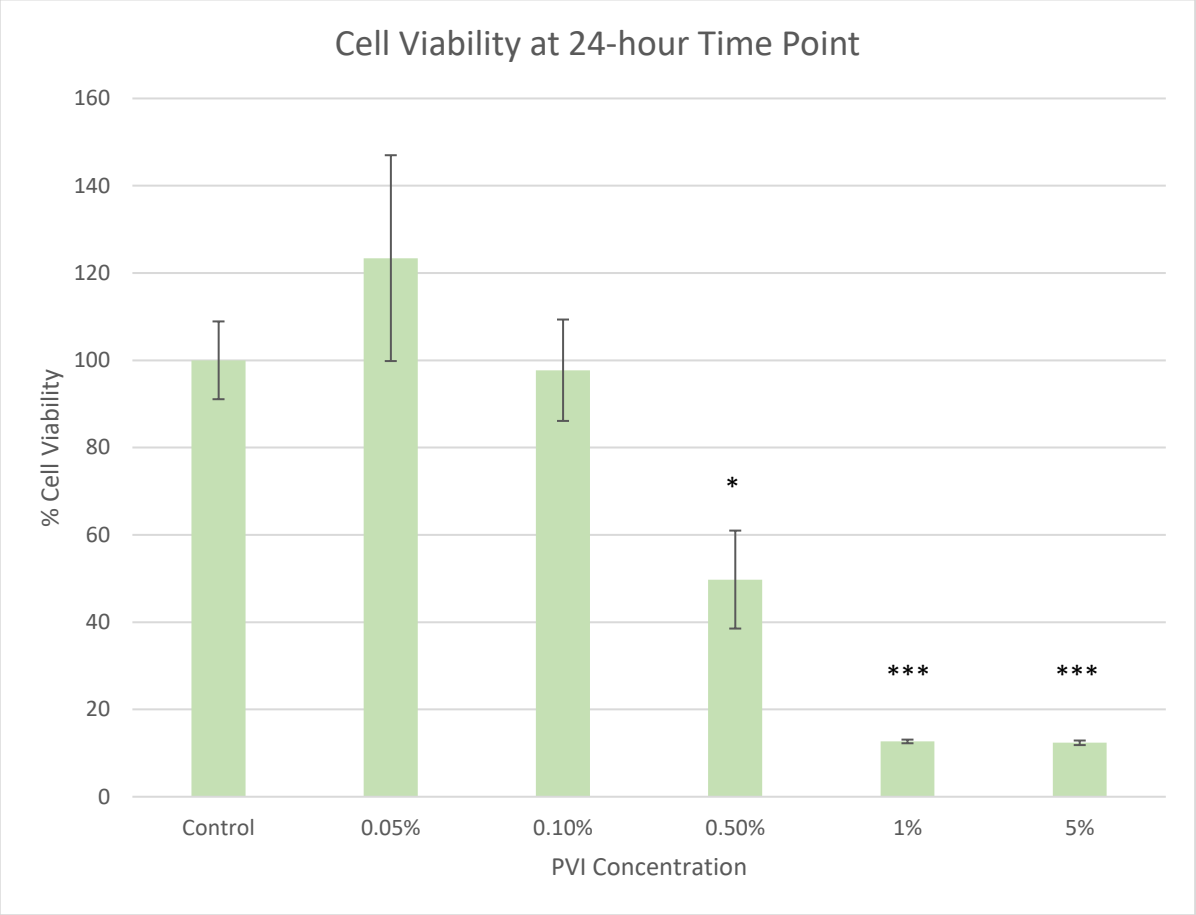

Figure 5-7. The cell viability was observed with 3 minutes of PVI exposure at 24-hour time point. Significant reduction was observed with the 0.5 %, 1 % and 5% concentrations with a Student t-test (**p<0.001, *p<0.05).**

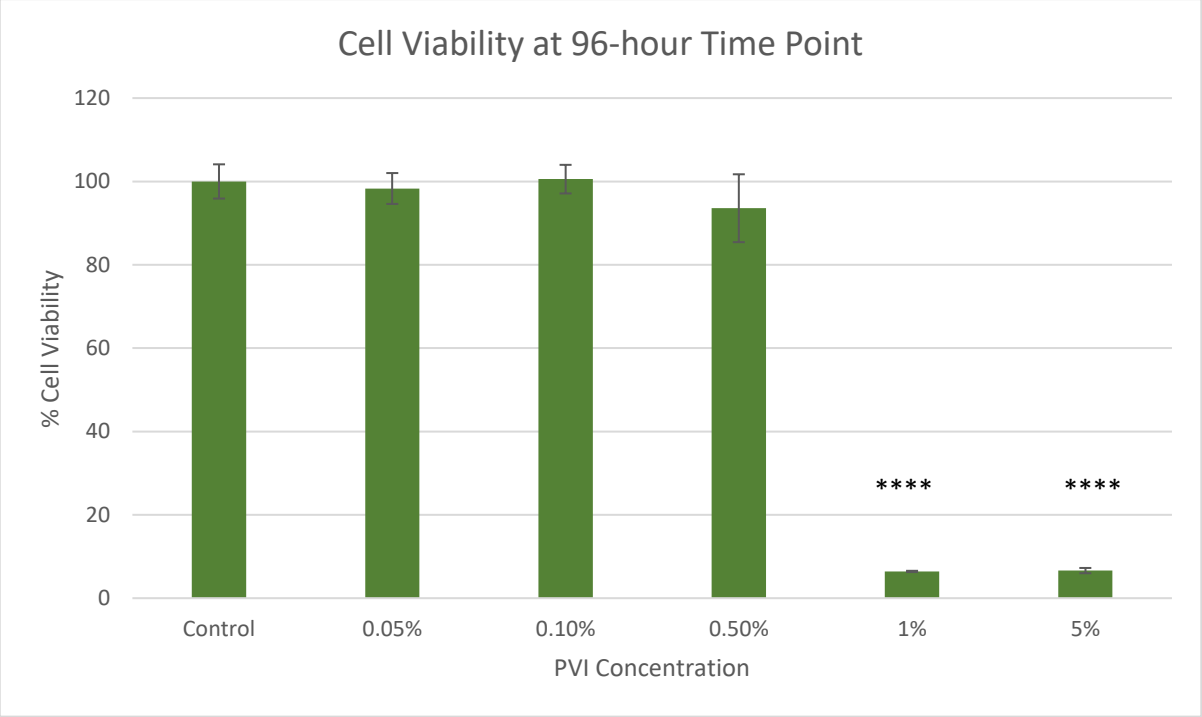

Figure 5-8. The recovery of the cells shown by cell viability at the 96-hour time point. Significant decrease in cell viability was still observed at 1% and 5% of PVI compared to control. Student t-test was performed. (**p<0.001).**

5.3.2 Imaging Results

Live/dead staining was carried out to observe the morphology of the cells and also to visualise their viability status. Cell viability was negatively affected in agreement with the results of the cell viability assays. Although the cells remained attached to the well plate where they had been seeded, most of them were stained dead as shown in Figure 5-9.

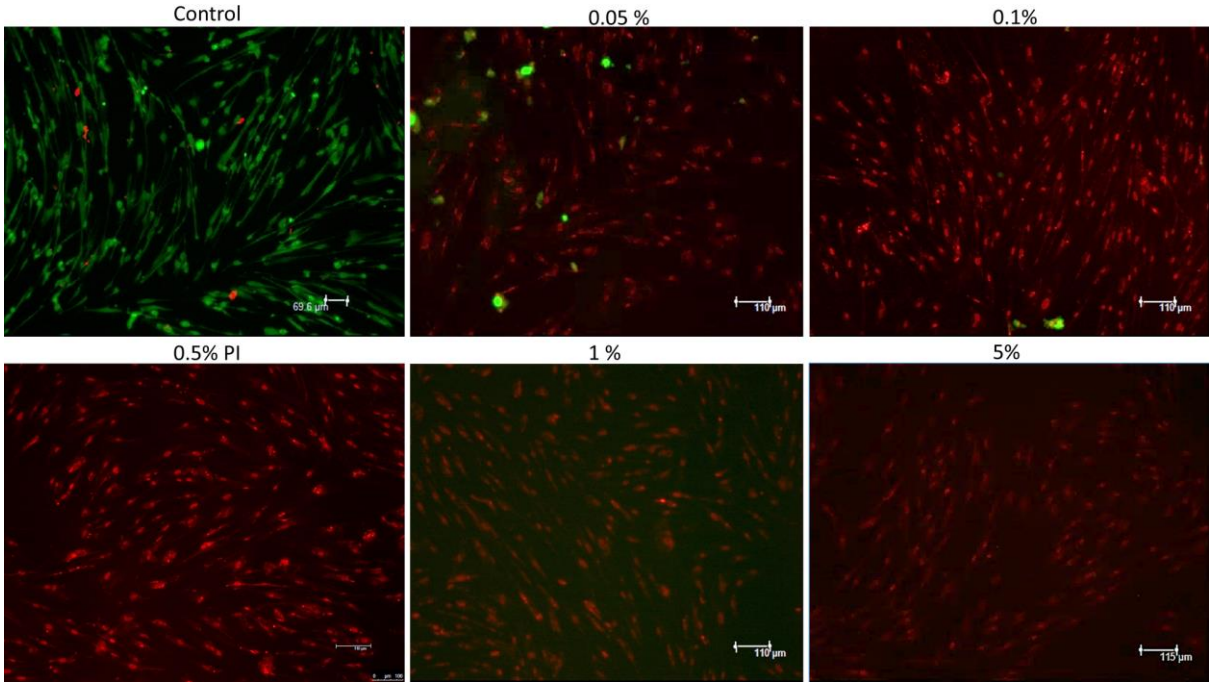

Figure 5-9. Live/Dead assay for different PVI (Nex Iodio) concentrations at 3-minutes exposure times. The analysis was carried out after 24 hours of culture. Dead cells are shown in red, live cells shown in green.

The scratch assay was also observed in order to monitor wound healing. Light microscopy images are shown in Figure 5-10 and these represent findings after 24-hour culture following the scratch assay and PVI exposure. Differences in cell migration to the wound are clearly apparent. The higher the PVI concentration, the larger the wound area.

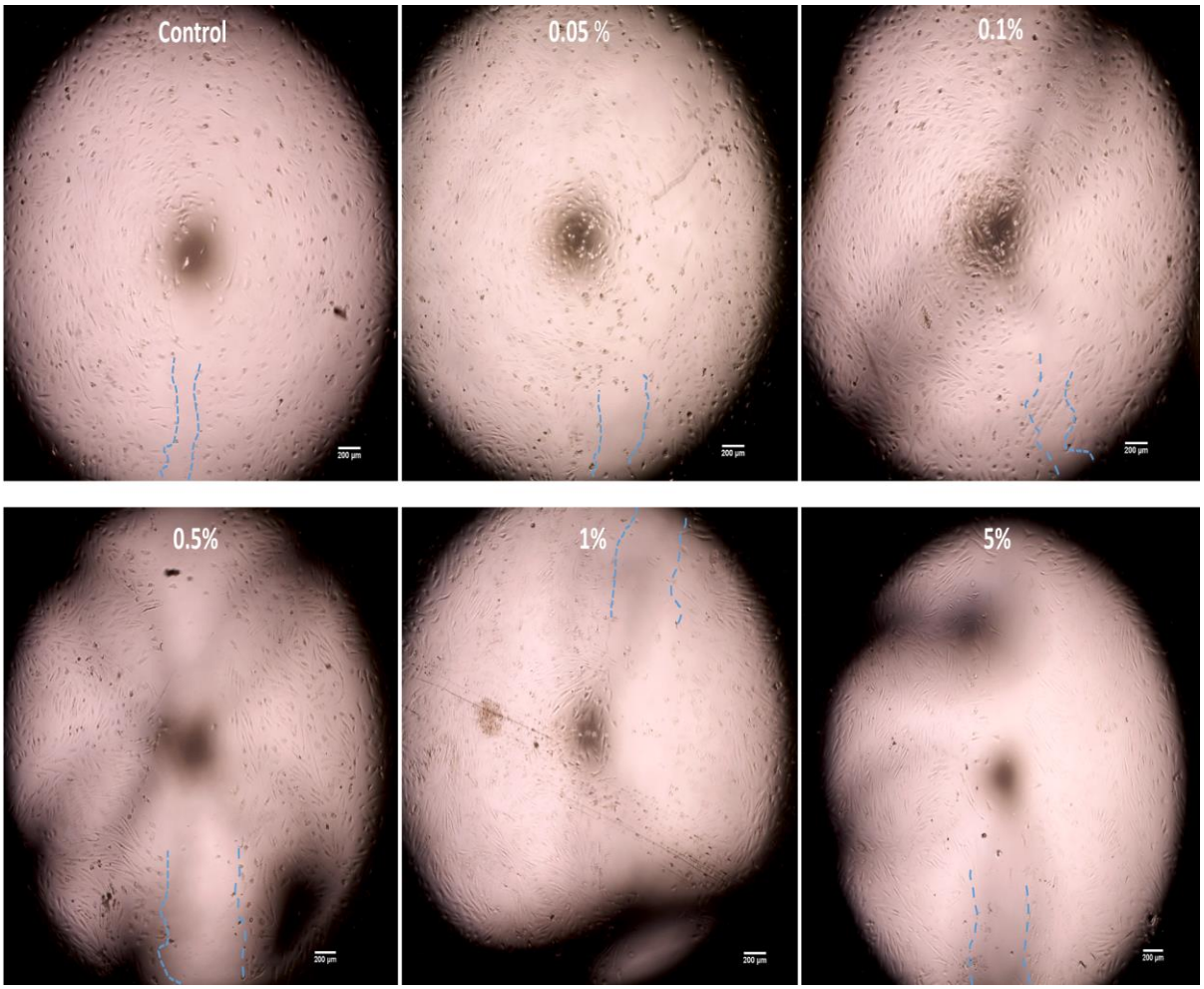

Figure 5-10. Representative images of the scratch followed with PVI (Minims) exposure for 3 minutes at 24-hour time point after. Images were taken 24 hours after the scratch was created with PVI (Minims) treatment. Blue arrows show the scratch area.

Live/Dead assay was carried out on the plates at the 24-hour time point. The alive and dead cells were clearly seen visible along with the wound in Figure 5-11. The dead cells dominated until concentrations lower than 0.5 % PVI were used, as expected from the proliferation assays in the previous section. At concentrations of 0.5 % PVI and lower, live cells started to appear however, the wound healing rate was still slower compared to the control (no treatment) wells. At 0.1 % PVI, migration can be seen with some cells populating the scratch area with smaller wound area than seen at the 0.5 % concentration. At exposures of 0.05 %, the wound was more closed than it was with any of the higher PVI

concentrations applied. On the contrary, there was almost no difference between the 'no treatment' and 0.05 % of PVI exposure, in terms of wound healing rate.

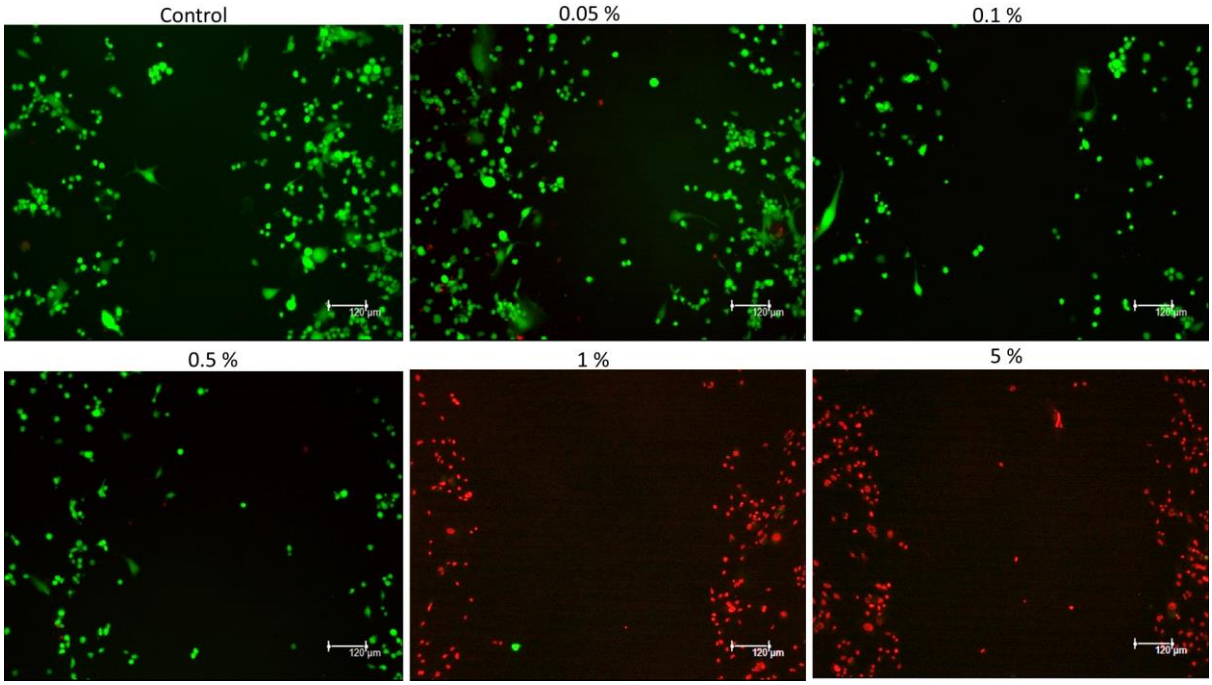

Figure 5-11. Visualised wound healing by the Live/Dead staining. The scratch was created just before the 3 minutes of PVI exposure. These images represent the cells which were cultured for 24 hours more after PVI exposure.

PVI was also exposed to collagen gel embedded with conjunctival fibroblasts in order to observe cell behaviour within a three-dimensional model in vivo. The concentrations from 5%, 1% and 0.5% were chosen from the previous results of this study. Collagen hydrogel results confirmed the monolayer results with a variety of concentrations of PVI seen in Figure 5-12. Only control and 0.5% of PVI exposure displayed predominantly the viable cells. The other concentrations in the hydrogel showed that the majority of the cells were dead. This also indicates that the PVI exposure for 3 minutes was able to penetrate inside of the hydrogel.

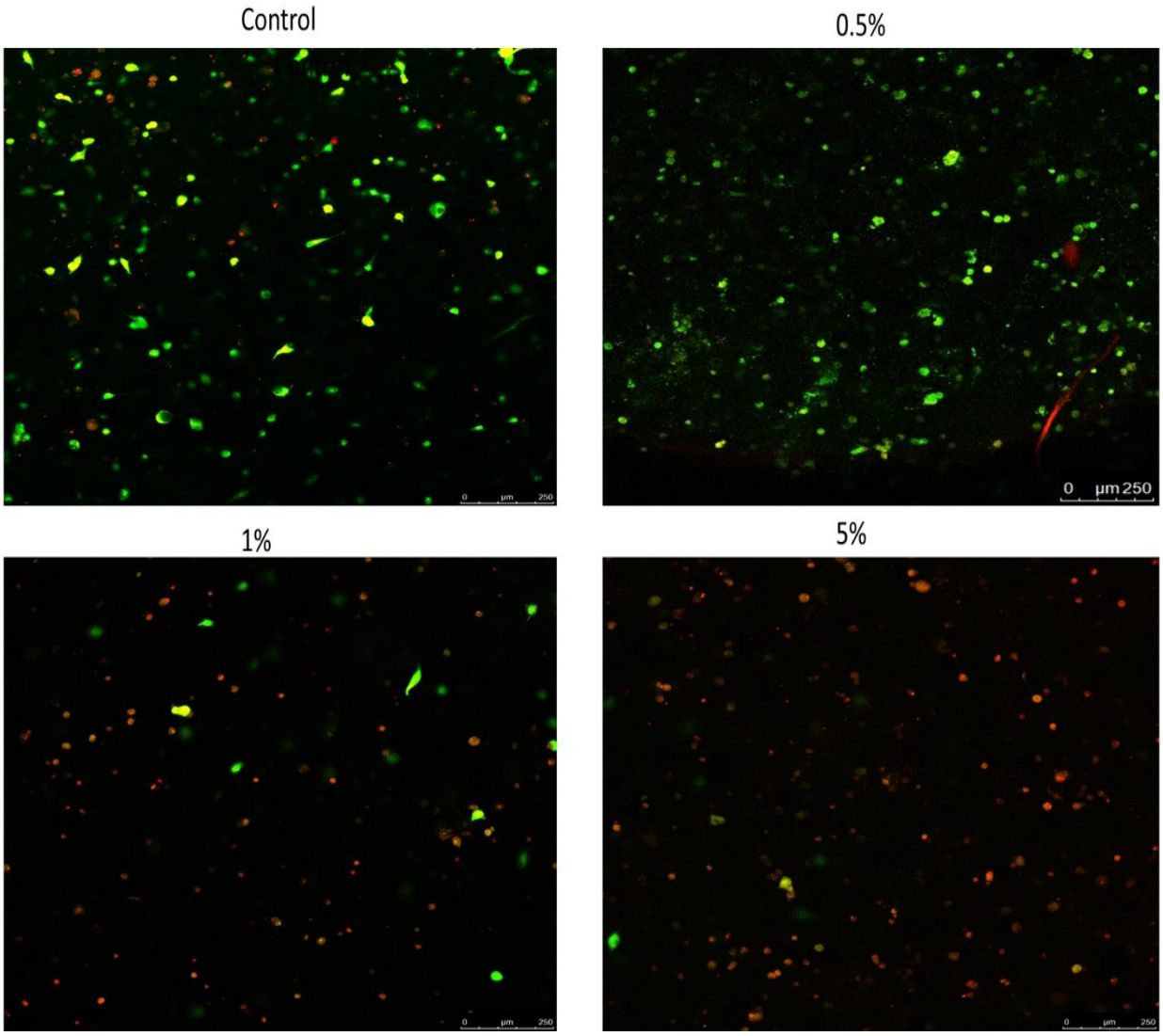

Figure 5-12. Visualized collagen hydrogel with different concentration of PVI exposure. Live/dead assay was performed to assess PVI exposure for 3 minutes on collagen gel cultured with conjunctival fibroblasts at 2- hour time point. Dead cells were shown to increase with rising concentration of PVI.

5.4 Discussion

The effect of PVI is important since it has become the standard of care in ophthalmic practice, especially before intravitreal injections and cataract surgery. The effects of PVI on the ocular surface have gained attention due to its possible association with dry eye signs and symptoms, often reported following ophthalmic surgery [101,102,147]. There are reports comparing tear film quality with and without the use of PVI. They observed that tear film quality was significantly affected by PVI intravitreal injection [128], corneal and conjunctival epithelial damage was also reported along with subjective complaints with the use of PVI as opposed to without the use of PVI [352]. Therefore, it has been considered as a risk factor for dry eye after the use of PVI during cataract surgery. Toxicity of PVI has

been investigated in vitro for ocular tissues; severe epithelial damage predominantly on the cornea was observed in terms of cell viability [201]. To date, limited research has been carried out on conjunctival damage by PVI exposure in vitro [203] even though conjunctival health is important for maintaining ocular surface health and homeostasis due to the presence of immune resident cells, and its contribution to the dry eye pathogenesis. Thus, this study investigated the effect of povidone-iodine toxicity and the effect of the wound healing on porcine conjunctival fibroblasts. To date, this is the first study reporting on the wound healing and toxicity of PVI using conjunctival fibroblasts.

In this study, the effect of PVI was assessed using cell viability and wound healing assays. Moreover, its penetration in three-dimensional cell culture with collagen gel was tested. The toxicity was found with higher than 0.5% of PVI concentrations in both two dimensional and three-dimensional culture conditions with the conjunctival fibroblasts. The cellular viability experiments revealed the influence of a variety of PVI concentrations and exposure times have a dramatic effect on conjunctival fibroblasts. Although PVI exposure is typically 3 minutes in surgical practice, this study investigated three minutes exposure along with the less than 3 minutes exposure times; 30 seconds, 60 seconds and 120 seconds in order to improve our knowledge of PVI mechanism of action. It was found that PVI affected the cell viability within less than 30 seconds.

This study also revealed the longer culture time period as 96 hours to observe the recovery rate of fibroblasts considering that the patient's ocular surface exposed to PVI can recover. The cell viability showed that the fibroblasts did not recover with higher than 1% of concentrations of PVI. Duration of PVI exposure also did not affect the recovery rate showing that toxicity of PVI is irreversible. Fibroblasts only displayed a small recovery with 30 seconds of exposure with 1 % of PVI. However, the cell viability was still significantly reduced at 96-hour time point with the 30 seconds exposure. More than 30 seconds of exposure, no recovery was observed in terms of cell viability which seems to be consistent with those of Chou et al [360] which investigated the response of the corneal cells for additional 5 days after 1 minute of PVI exposure (0.1-5%). No recovery was found after 96 hours time point after PVI exposure and led to a conclusion that the effect of PVI exposure is immediate, permanent and irreversible.

The adverse effect of PVI on cellular viability is seen using concentrations of 1% and above. The longer exposure time longer than 120 seconds only made a difference in the samples which were exposed to 0.5% of PVI. When the exposure is less than 3 minutes, such as 30, 60 and even 120 seconds, conjunctival cells were not affected by the 0.5% PVI exposure. Hence, less than 0.5% of PVI concentration was found to be safe for conjunctival fibroblasts for 3 minutes of exposure as a recommended exposure time.

In vitro toxicity studies of PVI exposure have been criticised by not being able to reflect the in vivo or clinical settings. Owing to the advantage of better mimicking in vivo conditions, three-dimensional models can offer a better environment to observe the actual body response. Collagen hydrogels are widely preferred to study the conjunctiva since the main matrix of the conjunctiva is made of collagen [244,361]. Similar behaviour of the cells to live/dead assay was observed as in the two-dimensional matrix which indicated the PVI penetration through the matrix. This study confirms that conjunctiva is permeable to PVI exposure, and can cause similar toxicity inside the collagen matrix even though PVI exposure is not as direct as than in two-dimensional cultures. Further quantitative and qualitative assessment of the PVI permeability is required to confirm the behaviour of the cells in three dimensional in vitro models. Moreover, owing to a variety of cell type in the conjunctiva, three-dimensional in vitro model with co-culturing of the cells would be ideal to study the conjunctival response.

These results are in line with those of, Bataille et al. [203], Yanai et al. [199] in vitro and Jiang et al [200] in vivo even though they used different cell types. In terms of toxicity level, similar findings have been reported in which 0.5% of PVI was found to be safe for corneal and conjunctival toxicity [200,362].

Form the live/dead imaging, PVI application induces the cell death through fixation as it can be seen in Figure 5-9 and Figure 5-10. Exposure to PVI caused loss of plasma membrane integrity which was stained by the Ethidium homodimer-1. Interestingly, PVI exposure which caused to cell death did not alter fibroblasts morphology. These results match with the study by Chou et al [360]. They found the same fixation effects on corneal epithelial and stromal fibroblasts with the PVI exposure from 0.1% to 5% for 1 minute. Exposed cells did not respond to any of the external stimuli even after 5 days.

It was also demonstrated that wound healing (after the scratch and PVI exposure) was delayed confirming that migration and/or proliferation of the conjunctiva fibroblasts was suspended by PVI exposure. Results of the cell viability gave some indication about cell migration and/or proliferation of the cells when the cells were closing the wound. The slower wound healing rate was observed at 0.1% and 0.5% of PVI concentration even though the cells were found to be predominantly alive. These findings revealed that PVI affects the fibroblast's proliferation or migration or both even though they were shown to be viable when they are exposed to those concentrations of PVI. Compared to no exposure to PVI, with 0.05% of PVI concentration did not show any difference in terms of closing the wound. With this study, it can be confirmed that the higher than 0.05% of PVI exposure delayed the wound healing however, it is not certain whether the cell proliferation or cell migration or both were adversely affected by PVI exposure. Further studies are required to investigate the migration and proliferation phenomena to understand the exposure mechanism better.

A considerable amount of literature has been published on the effect of PVI on wound healing. Clinical experience and in vivo studies along with in vitro studies have been trying to reach a consensus about PVI concentration. In clinical settings, PVI is generally considered as a safe antiseptic which does not cause a delay in wound healing. Fibroblasts have been the main focus in most of the wound healing studies due to its function in producing pro-inflammatory mediators during injury and inflammatory stimuli [363]. In fact, there is a growing body of literature regarding the use of PVI in the treatment of acute wounds, surgical wounds, burns and chronic wounds due to its efficacy of bactericidal activity [229,364]. Thomas et al. [118] were one of the first groups to explore more than cytotoxicity of PVI of dermal fibroblasts in which they observed complete attenuation on fibroblasts migration and proliferation by leaving 0.2% of PVI in the culture media 24 hour time. The slower wound closure was observed within time 0, 24 and 48 hours' time points. Recently, Liu et al. [349] found that myoblasts, osteoblasts and fibroblasts' migration and viability was adversely affected using a concentration of PVI greater than 0.1% with a 3-minute exposure. Even though no study has tested conjunctival fibroblasts, these results are in agreement with this study which showed that higher than 0.05% of PVI concentration caused a delay in wound healing response of fibroblasts.

Even though wound healing is crucial after the cataract surgery, the previous literature has not addressed the wound healing response affected by PVI exposure on the ocular surface. Not only cataract surgery but most of the ophthalmic surgery caused denervation on the ocular surface leading to impairment of wound healing which might be one of the reason for dry eye postoperatively [147,155]. Hence, it is crucial to choose antiseptics which do not add additional stress to the ocular surface for wound healing where possible.

Furthermore, two different PVI preparation were tested for toxicity studies in terms of cell viability. Even though the dilutions were prepared in the same way, the cell viability was affected differently with two PVI exposures. Nex Iodio P2 PVP exposure caused more adverse toxicity than the Minims PVI. Despite the fact that the purpose of the use of the two PVI is different from each other; Nex Iodio for washing hands for preoperative procedures and Minims for ophthalmic applications before the surgery, the similar responses from the conjunctival cells with the same concentration of solutions were expected. This could be due to variation in free iodine level in the solution. Iodine chemistry is still not understood completely due to its complexity and measuring the free iodine in a solution is still a controversial [197]. Iodine's deleterious effect has been shown for the first time on conjunctival fibroblasts in this study with greater than 0.5% of PVI in terms of cell viability. To date, no validated in vitro model exists to test and compare PVI toxicity, therefore there is still more research required to understand the lowest toxicity level which maintains the effective iodine activity. In conclusion, PVI is a very commonly used antiseptic agent not only in dermatology but also in the ophthalmology applications with preoperative or postoperative use. Although there are a few studies investigating the toxicity of the povidone-iodine on the ocular surface, mostly on the cornea, only a few studies exist in scientific literature investigating the effects on the conjunctival epithelial cells. To the best of our knowledge, this was the first time conjunctiva fibroblasts toxicity was reported in the scientific literature. This study indicated the concentration PVI greater than 0.05% slowed down wound healing in this monolayer and caused a significant decrease in viability in both two dimensional and three-dimensional culture model with conjunctival fibroblasts. This study has important ramifications in the understanding of complaints, discomfort and dry eye signs often seen after cataract surgery and

further study is required to confirm the toxicity mechanism in order to modify povidone iodine use to reduce the side-effects on the ocular surface.

CHAPTER 6: LIMITATIONS AND FUTURE DIRECTIONS

6 LIMITATIONS AND FUTURE DIRECTIONS

Overview

The ultimate goal of the thesis was to enhance the understanding of the impact of cataract surgery on ocular surface homeostasis and its association with dry eye by investigating different aspects applied used before, during or after the surgery using an in vitro experimental approach. Porcine conjunctival fibroblasts were used to establish a dry eye in vitro model in which hyperosmolar stress was used to stimulate the dry eye condition. Whilst this thesis adds testament to the influence of light exposure and povidone iodine on conjunctival fibroblasts, further work is required to ultimately translate the findings into the clinical settings.

6.1 Overall Limitations

This thesis focused on assessing the damage to conjunctival cells due to a combination of factors relevant to cataract surgery, in order to elucidate the molecular aspects of the development of the postoperative dry eye. The conjunctival in vitro model developed in this thesis was designed to closely mimic the in vivo condition of dry eye, however differences between in vitro and in vivo conditions naturally exist.

First of all, hyperosmolar stress and the wound healing response were central aspects of this thesis used to assess conjunctival damage with a combination of other factors in order to improve understanding the pathology of dry eye after cataract surgery and its reasons in a molecular way.

One of the limitations is that one type of conjunctival cells was used for this research while the conjunctiva is housing a variety of cells in vivo (epithelial cells, goblet cells and inflammatory cells). Fibroblasts were chosen since there was a limited number of studies published in the scientific literature investigating the role of conjunctival fibroblasts in dry eye disease, despite their key role in the inflammation response to external stressors [365]. Therefore, it was not possible to assess the co-operation between the different conjunctival cell types in conjunctiva for the stressors used in this thesis.

Secondly, the use of porcine tissue is arguably a further limitation. On one hand, porcine eyes have been shown to more closely resemble human eyes than rabbits and mice eyes [153,173,244,366]. However, a very limited amount of information regarding animal age, sex and health status is available when using porcine eyes obtained as a slaughterhouse(secondary) waste products [367]. In this thesis, this problem was minimised as eyeballs were personally collected by trained personnel of Aston University that selectively collected fresh tissue before the scalding procedure.

Finally, the in vitro model developed in this thesis was based on conjunctival primary cells due to their ability to retain their morphology, biomarkers and biochemical functions in vitro [368]. However, they are characterised by a short-life span and limited repeatability. To overcome this limitation further studies could be conducted using cell lines, which instead offer a much greater life span and repeatability, however, cell lines do not necessarily represent accurately the primary cell function and also it has been debated for the potential contamination [220]. But it will be worth to try and observe the response of the conjunctival cell lines.

6.2 Operating Microscope

Chapter 3 attempted to investigate the effect of the operating microscope light on conjunctival fibroblasts by assessing wound healing, IL-6 levels, cell viability and apoptosis rate in vitro.

The light from the operating microscope led to a decrease in cell viability, increase in IL-6 levels and slower wound healing when the conjunctival fibroblasts were exposed to high hyperosmolarity stresses. This preliminary study suggests that an operating microscope can cause phototoxicity on stressed cells by affecting different cellular metabolic mechanism. This is reinforced by the fact that a light exposure of 10 minutes was chosen throughout the thesis to mimic exposure due to cataract surgery performed by an experienced surgeon [270]. Hence, the ideal surgery situation was studied to assess the minimal time exposure to the operating microscope. This result could have a clinical relevance since a preoperative dry eye can exacerbate the negative effects of light exposure on the ocular surface. However, further in vivo studies are required to investigate this aspect further.

In addition, the photothermal effect of the surgical microscope was assumed to be negligible in this experimental setup. This is due to the exposure time being shorter than the threshold needed to cause any thermal effect on cellular behaviour [369]. Although it is believed that heating of the system was not observed with this experimental set up since the experiments on the well plate were carried out with the lid on, further studies could be conducted measuring the sample temperature with a thermal camera (thermography) to quantitatively characterize the system.

Finally, the in vitro findings of this chapter could be expanded by performing a more detailed molecular analysis of light exposure such as PCR. A wider molecular analysis could help to discriminate between different light irradiation in terms of power and wavelength that could potentially lead to the development of optimised filters to reduce the phototoxicity further.

6.3 UV Irradiation

Whilst a few studies have shown that UV light can cause damage to the ocular surface and few have investigated the association with dry eye [168,266]. In particular, ocular surface cells exposed to hyperosmolar stress were shown to respond more adversely to UV-A radiations in terms of cellular viability [168]. Based on these findings, an in vitro study was designed in this thesis to observe the effect of different UV wavelengths on conjunctival fibroblasts exposed to hyperosmolar stress. An adverse reaction in terms of cell viability was found when UV-A with 370nm wavelength was shone on

the cells for 10 minutes. The main limitation of this study is due to the different energy levels of different UV radiations which makes it difficult to compare the effects of different filters. Therefore, it would be beneficial to study which wavelength has the greatest effect on conjunctival fibroblasts by balancing exposure time and energy level of the UV source. Furthermore, a detailed molecular approach is necessary to fully understand the relationship between UV radiation and cellular health. More quantification of cellular behaviour would facilitate the comparison of UV radiation with average environmental exposure, operating microscope exposure and ultimately clarify the link between dry eye disease and UV exposure.

6.4 Povidone Iodine Exposure

Although there are many studies reporting on the toxicity of PVI exposure, PVI is highly effective and commonly used clinical antiseptic agent. Laboratory studies on povidone iodine suffer from the lack of a validated in vitro model and, therefore, the concentration used and exposure time vary greatly among studies. Therefore, in this thesis, the effect of different exposure times and PVI concentrations was assessed on conjunctival fibroblasts both in two dimensional and three-dimensional culture. Three-dimensional culture also indicated the efficacy of PVI to penetrate inside the matrix mimicking the in vivo conditions of conjunctival fibroblasts. Co-cultured conjunctival cells in a three-dimensional culture would be useful to explore PVI toxicity on the conjunctiva.

This study is the first of its kind and it was hypothesized that conjunctival fibroblasts are directly exposed to povidone iodine. However, during cataract surgery, the conjunctiva could not be directly exposed to PVI when there is healthy epithelium on top of the fibroblasts. [197,370]. However, dry eye patients, for instance, might not have a healthy epithelium which makes conjunctival fibroblasts susceptible to be affected with such agents. Therefore, further studies should be conducted to take this limitation into consideration.

CHAPTER 7: CONCLUSIONS

7 CONCLUSIONS

Advancing modern medicine requires a multidisciplinary approach including the use of in vitro models that could investigate physiological mechanisms of several pathologies more easily. In this framework, this thesis generated an in vitro ocular model that allowed authors to unveil key information about the effect of cataract surgery on conjunctival health. This study is clinically important as cataract surgery is the most performed surgery in the world and the prevalence of dry eye after cataract surgery has been estimated to reach as much as 70% [3]. A better understanding of postoperative dry eye is likely to dramatically improve patient's satisfaction after cataract surgery.

This is the first time light from an operating microscope, UV irradiation and povidone iodine have been investigated in vitro along with the association of dry eye using conjunctival fibroblasts. This study gives valuable information that is not only applicable to cataract surgery but also for other ocular surgeries. Light from the operating microscope can adversely affect wound healing, induce apoptosis and reduce the viability of cells under hyperosmolar stress.

UV-A damage on conjunctival fibroblasts under hyperosmolar stress was observed. Different radiation with different wavelengths was tested, and conjunctival fibroblasts were found to be more sensitive to high energy UV-A radiations when stressed. This mechanism may elucidate why people affected by

dry eye are found to be more sensitive to UV irradiation either from environmental sources or perhaps from operating microscopes than are healthy subjects.

PVI toxicity on conjunctival fibroblasts found that PVI concentrations higher than 0.5% completely froze down the cellular wound healing mechanisms. In the clinical setting, PVI might not come into direct contact with the conjunctival fibroblasts prior to surgery, however, this study showed that direct leakage of PVI onto ocular surface could adversely affect conjunctival health in less than 30 seconds.

In this thesis, in vitro model of dry eye was developed in an attempt to investigate the possible impact of the aspects of ophthalmic surgery on ocular surface cells with a reliable and cost-efficient approach. Specifically, this research was the first to report the adverse impact of aspects surgery on conjunctival fibroblasts, which contributory factor in postoperative dry eye development and/or worsening often reported in the literature. Further in vitro research on other conjunctival and corneal structures and ex vivo models along with clinical investigations will help to translate the knowledge gained into the clinical settings.

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